PHASE CONTROL OF THE CIRCADIAN RHYTHM OF CO_2 EXCHANGE IN LEAVES OF *BRYOPHYLLUM FEDTSCHENKOI*

by

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SUMMARY

An investigation has been made of the generation and phase control of the circadian rhythm of carbon dioxide exchange in leaves of *Bryophyllum fedtschenkoi*.

In continuous light, and a stream of normal air at 15°C, this rhythm is very marked and persists for many days with a period of 18.2 ± 0.16 h. The rhythm operates within the approximate temperature range 10 - 30°C, the period becoming longer with increasing temperature. Outside this range the rhythm is inhibited but can be reinitiated by restoring the temperature to 15°C.

Prolonged exposure of the leaves to high and low temperature appears to inhibit the rhythm by forcing the basic oscillator to, and holding it at, fixed phase points in the cycle which differ by 180°. These phase points have been characterised in terms of the malate status of the leaves. High temperature inhibits the rhythm in such a way that little or no fixation of CO_2 occurs and the leaves have a low malate status. Low temperature allows the leaves to fix substantial amounts of CO_2 and, as a consequence, they have a high malate status.

The rhythm of CO_2 exchange exhibited by leaves maintained in light and normal air is also inhibited by prolonged exposure of the leaves to darkness and to 5% CO_2, but oscillation begins from a fixed phase point when leaves are transferred to light and normal air respectively. The nature of the inhibition of the rhythm brought about by darkness and 5% CO_2 appears to be similar. Both of these treatments result in the leaves accumulating relatively large amounts of malate, suggesting that, in common with low temperature, darkness and 5% CO_2 inhibit the rhythm by holding the oscillator in a malate-rich state.

Brief exposure of the leaves to high and low temperature, darkness and 5% CO_2 shifts the phase of the rhythm, the magnitude of the phase shift depending upon the position in the cycle at which the treatment is applied. Phases shifts were not

found to be accompanied by a detectable change in the gross malate status of the leaves. A fixed relationship was found to exist between the time in the cycle at which a short high or low temperature treatment ended and the time of occurrence of the next peak of the rhythm. Such a relatioship was not always found for short dark or 5% CO_2 treatments. Nevertheless the oscillator appeared to be sensitive to high temperature in those positions in the cycle at which it is insensitive to low temperature, darkness and 5% CO_2 and vice versa.

An oscillation in the malate content of leaves maintained at 15°C in continuous illumination and a stream of normal air was observed. This oscillation was discernable for up to 72 h but damped out rapidly with time, in contrast to the CO_2 exchange rhythm which persisted for many days under such conditions.

It was concluded that if malate synthesis and breakdown are involved in the generation and phase control of the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi*, then it is localised changes in the malate concentration within a cellular compartment rather than the gross malate status of the leaves which are of critical importance.

ABBREVIATIONS

ATP	Adenosine Triphosphate
CAM	Crassulacean acid metabolism
DD	Continuous darkness
ESA	European Space Agency
GOT	Glutamate oxaloacetate amino-transferase
LL	Continuous light
MDH	Malate dehydrogenase
NAD	Nicotinamide adenine dinucleotide
NASA	National Aeronautics and Space Administration
N.S.	Not significant
OAA	Oxaloacetic acid
PEPCase	Phosphoenolpyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
ppm	Parts per million
RuBP	Ribulosebisphosphate
S	Level of significance
UV	Ultraviolet radiation
*	Significant at 5% probability level
**	Significant at 1% probability level

GLOSSARY

PERIOD: time between the repetition of a definite phase point in an oscillation. Usually the distance between the peaks or troughs of a rhythm are measured.

FREE-RUNNING PERIOD: period exhibited by an organism maintained in a uniform environment.

PHASE: instantaneous state of an oscillation within a period.

PHASE SHIFT: Displacement of an oscillation along a time axis.

ENTRAINMENT: Coupling of an endogenous oscillator to periodic variation in an external parameter.

ZEIGEBER: Time cue or synchroniser.

1. INTRODUCTION

1.1. HISTORICAL BACKGROUND

The capacity of living organisms to measure the passage of time and to regulate their biochemical, physiological and behavioural processes on a temporal basis has been recognised for more than 200 years. Although reports of the periodic movement of leaves date back to the first century A.D., it was the definitive experiment of the Dutch astronomer, De Mairan, in 1729, which first focused attention on the apparent capacity of a plant to continue the rhythmic movement of its leaves in the absence of periodic environmental signals. This finding strongly indicated the presence in the plants of an endogenous self-sustained oscillator which might serve as the basis of time measurement. Almost two centuries were to elapse, however, before the most essential feature of the oscillator required for accurate time measurement, namely the near temperature independence of the period or frequency, was established. The wide-spread occurrence of temperature-compensated, persistent, circadian rhythms in plants, animals and humans is only one of several manifestations of the operation of a time-measuring mechanism or biological clock in living organisms. The existence of phenomena such as photoperiodic responses and solar navigation also demand accurate time-keeping. It is however, with the study of endogenous circadian rhythms that this thesis is wholly concerned.

The term circadian is derived from the latin circa meaning about and dies meaning day, and was first suggested by Halberg (1963) to define those rhythms which persist under constant environmental conditions with a period of approximately 24 h. It is outwith the scope of this introduction to provide a complete list of definitions for all the terms encountered in the field of circadian

rhythmicity, but comprehensive lists are provided in the books by Aschoff (1965) and Brady (1982) and the terms used in this thesis are explained in the glossary.

Most, if not all, early studies of rhythmic processes were concerned with establishing their endogenous nature. The experiments of Duhumel in 1758 demonstrating the continuation of leaf movement in the relatively uniform environment of caves, and those of Zinn, a year later, confirmed the earlier results of De Mairan and focused attention on the possible existence of endogenous periodic phenomena in plants. Many eminent biologists including Darwin, Hofmeister, Sachs and Pfeffer became interested, during the latter part of the 19th century and the early part of this century, in the periodic movement of leaves, a rhythm which provided the basis for many studies during this period because of the ease with which it could be measured. Studies of rhythmic phenomena were not, however, confined to plants. Kiesel for example, in 1894, discovered that arthropod pigmentation cycles persisted under constant conditions.

By the end of the 19th century the existence of an apparently endogenous, self-sustained oscillator had been demonstrated on a number of occasions in both plants and animals. The function of such a mechanism however, does not even appear to have been considered until the early years of this century when Bünning, during his extensive studies of leaf movement in *Phaseolus multiflorus*, suggested that endogenous rhythms may be used by organisms to measure the passage of time (see Bünning, 1960). Bünning discovered that the period of oscillation in this rhythm increased slightly with increasing temperature indicating that the oscillator may be of a metabolic nature. He pointed out that in contrast to other metabolic processes where a 10^{0}C rise in temperature doubles or even triples the rate of a reaction, the frequency of oscillation in *Phaseolus* changed only very slightly with temperature. For an oscillating system to provide an accurate means of time-measurement it must be capable of oscillating with the same frequency despite fluctuations in ambient temperature. It was the possession of this essential feature by circadian rhythms that Bünning demonstrated so

significantly, although at the time he was perhaps not fully aware of the importance of his observation. The full implication of his discovery was not generally appreciated until the publication, in 1954, of Pittendrigh's paper on the rhythm of eclosion in *Drosophila* in which the importance of a temperature-compensated period was placed fully in perspective as providing the basis for the biological clock or time measuring mechanism in living organisms.

1.2. THE OCCURRENCE OF RHYTHMS

The realisation that living organisms possessed a mechanism enabling them to measure the passage of time initiated a new period of interest in biological rhythms. Early studies had necessarily been restricted to those rhythms which could be easily measured automatically with mechanical devices such as kymo-graphs, but technological advances later enabled rhythms to be monitored in a wide variety of biochemical, physiological and behavioural processes in eukaryotic organisms at all levels of organisation.

A number of unicellular and multicellular plants exhibit circadian rhythms which have been subjected to detailed investigation. Amongst the lower plants are the rhythms of luminescence in the photosynthetic, armoured, marine dinoflagellate, *Gonyaulax polyedra* (Hastings and Sweeney, 1958), of phototaxis in *Chlamydomonas reinhardi* (Bruce, 1970) and *Euglena gracilis* (Bruce and Pittendrigh, 1956), of photosynthesis in *Acetabularia major* (Sweeney and Haxo, 1961) and of sporulation in *Oedogonium cardiacum* (Bühnemann, 1955a). In the fungi rhythms in growth rate and conidiation in *Neurospora crassa* (Sargent, Briggs and Woodward, 1966; Pittendrigh, Bruce, Rosenweig and Rubin, 1959), and in sporangium discharge in *Pilobolus sphaerosporus* (Uebelmesser, 1954) have received particular attention, as has the rhythm of spore discharge in *Daldinia concentrica* (Ingold and Cox, 1955).

There are seemingly no reports of circadian rhythms in the Bryophytes and

only one in the Pteridophytes, that of the change in plastid shape in *Selaginella serpens* (Busch, 1953).

In higher plants a large number of rhythms have been reported. Those of leaf movement in *Phaseolus multiflorus* (Bünning, 1958), *Albizzia julibrissin* (Satter and Galston, 1971a) and *Samanaea saman* (Palmer and Asprey, 1958a) have been extensively studied, as has that of petal movement in *Kalanchoë blossfeldiana* (Bunsow, 1953). Rhythms also occur in the growth rate of the coleoptiles of *Avena sativa* (Ball and Dyke, 1954) and in the rate of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* (Wilkins, 1959) and *Kalanchoë daigremontiana* (Nuernbergk, 1961; Buchanan-Bollig, 1984).

Circadian rhythms have, therefore, been detected in processes as diverse as leaf movement, growth, CO_2 exchange and, more recently, nucleic acid synthesis in *Gonyaulax polyedra* (Waltz, B., Waltz, A. and Sweeney, 1983) and *Pisum sativum* (Kloppstech, 1985).

With one possible exception there are no reports of endogenous rhythms in prokaryotic organisms. The exception is *Escherichia coli* in which a rhythm in growth rate has been reported (Rogers and Greenbank, 1930).

Space does not permit an exhaustive review of the very large numbers of studies of circadian rhythms in plants which have been carried out since the resurgence of interest in this field in the middle years of this century. There are a number of excellent books by Bünning (1973), Sweeney (1969) and Brady (1982), and reviews by Mansfield and Snaith (1984) and Johnson and Hastings (1986), where such information is readily available.

1.3. ENDOGENOUS/EXOGENOUS ORIGIN OF RHYTHMS

Over the years a major area of controversy has been the question of whether circadian oscillations are generated from within the organism, or whether they are, in reality, the response of the organism to periodic variation in subtle environmental factors. The possibility of an exogenous factor being responsible for the generation of these rhythms was propounded principally by the late F. A. Brown jr. and his colleagues. Brown (1965) argued that the continuation of a rhythm in a so-called uniform environment may in fact be due to the response of the organism to periodic stimulation by an uncontrolled environmental parameter such as barometric pressure, the intensity of the earth's magnetic field or cosmic ray bombardment. In an extensive study investigating the effects of subtle geophysical factors on a wide variety of circadian systems, Brown (1962, 1969) reported that the rhythms in organisms as diverse as *Fucus*, carrot, bean seedlings, fiddler crabs, oysters and rats could be correlated with periodic changes in the intensity of the earth's magnetic field. There developed during the period from 1960 to 1970 a very substantial controversy on this matter between the school of Brown and most other investigators in the field. A number of attempts have been made to resolve this controversy.

Evidence against the possibility of an exogenous factor being responsible for the generation and timing in circadian rhythms was obtained from experiments carried out near the geographic South Pole (Hamner, Sirohi, Hoshizaki and Carpenter, 1962). Organisms were placed on turn-tables revolving in the opposite direction to the rotation of the earth, thus eliminating all possible diurnal stimulation except that due to the rotation of the magnetic pole around the geographic pole. Under such conditions rhythmic behaviour was displayed in a number of physiological and behavioural processes in a wide variety of organisms including bean seedlings, fungi and fruit flies. Further experiments indicated that these rhythms were not affected by magnetic fluxes many times greater than the strength of the earth's magnetic field, making it unlikely that the rhythms had

been influenced by the rotation of the magnetic pole round the geographic pole. A number of rhythms are, however, known to be influenced by magnetic fields. It has recently been reported, for example, that the enzymes responsible for diurnal melatonin biosynthesis in quails are affected by a decrease and increase in the strength of the earth's magnetic field (Cremer-Bartels, Krause, Mitoskas and Brodersen, 1984). These authors conclude that changes in the magnetic field strength of the earth are not responsible for driving the rhythm, but may act as additional zeitgebers.

If rhythmicity in an organism is a result of 24-h periodic stimulation by a subtle environmental factor then the period of the oscillating system would be expected to be precisely 24 h, as it is in rhythms in organisms exposed to the natural cycles of day and night. One of the major difficulties with the exogenous theory of rhythmic generation is the fact that most circadian rhythms have free-running periods which differ significantly from 24 h. As early as 1832 De Candolle noted that the period of the rhythm of leaf movement in *Mimosa* was 22-23 h and since this early discovery the free-running periods of most rhythms have been found not to be exactly 24 h, but rather values of between 18 and 30 h depending upon the organism and the precise nature of the constant conditions in which it is held. Brown (1969) has attempted to explain the occurrence of non-24 h, free-running periods by a mechanism which he terms autophasing. This mechanism is based on the observed cyclical variation in the sensitivity of organisms to phase-resetting signals such as light and temperature. Thus, the phase of a rhythm which persists in constant light or constant darkness will be shifted when the light sensitive or dark sensitive part of the cycle is reached and, depending upon whether the phase is advanced or delayed, shorter or longer periods will result. A similar principle would apply to explain variations in a period which changes with ambient temperature.

In order to establish unequivocally the endogenous nature of a rhythm it would be necessary to demonstrate its continuation in an environment in which there

was no possibility of the organism being exposed to 24-h periodicities. Such conditions pertain in orbiting space satellites and especially in deep space probes. Several studies of rhythms in space have been attempted but the results are reported only very briefly in the internal documents of the space agencies and are not therefore generally available. One study which is reasonably well documented concerns the rhythm of conidiation in the fungus *Neurospora crassa* which was found to continue for the 7-day duration of the orbiting Spacelab mission SLS-1 of NASA (Sulzman, Ellman, Fuller, Moore-Ede and Wassmer, 1984). Unfortunately, the cultures were accidentally exposed to light at least once during the space flight, and the effect of this stimulus on sustaining the rhythm could not be assessed. Repetition of this experiment is urgently required. Present Spacelabs launched by NASA and ESA orbit the earth once every 90 minutes and a potential difficulty arises because some rhythmic organisms may have the capability of demultiplying frequencies. Frequency demultiplication is a special type of entrainment in which the period of the observed rhythm is an exact multiple of the entraining cycle, rather than being equal to it as in normal entrainment. Frequency demultiplication has been observed in a wide variety of plants. For example, the period of the rhythm in leaf movement in *Phaseolus multiflorus* can be entrained to 28 and 32 h by exposing the plants to light-dark cycles of 14 and 16 h respectively (Flügel, 1949). Continuation of a rhythm in an orbiting satellite is good evidence for the endogenous nature of that rhythm provided the organism in question can be shown not to exhibit frequency demultiplication. One space experiment in which this possibility appears to have been eliminated concerns the rhythm of phototaxis in the alga *Chlamydomonas reinhardi*. This organism was taken on board the D1 Spacelab mission, and the rhythm was found to continue in space with a period similar in length to that of control organisms monitored on the ground during the flight. Moreover, the rhythm exhibited by organisms in the space lab had a larger amplitude and showed a greater degree of persistence than that exhibited by control organisms on the ground (personal communication to

Prof M.B. Wilkins from Prof D. Mergenhagen, University of Hamburg, Germany). Although this experiment has yet to be repeated, the results would appear to provide very good evidence that circadian rhythms are not dependent on external time cues, and must therefore be of endogenous origin. Final proof of this view must await the demonstration of the persistence of circadian rhythms in organisms in non-orbiting, deep space probes.

There is, in reality, very little evidence to support the exogenous theory for the generation of circadian rhythms. It is now generally accepted that a rhythm can be described as endogenous if it conforms to five conditions suggested by Pittendrigh in 1954. These are as follows:

1. The rhythm must persist in an environment in which as many environmental conditions as possible are held constant.

2. It should be possible to initiate a rhythm by a single stimulus.

3. It should be possible to shift the phase of the rhythm to any time of day and the new phase should be retained under constant conditions.

4. Hypoxia should stop the oscillator.

5. The free-running period should not be precisely 24 h.

It will become evident in the next section where the physiological characteristics of a number of rhythms are described that all rhythms discussed in this thesis conform to these five conditions.

1.4. PHYSIOLOGY OF RHYTHMS

Whilst it is now generally accepted that environmental periodicities are not responsible for the generation of circadian rhythms it will become apparent in this section that external stimuli influence endogenous rhythms by acting as time cues or zeitgebers. The physiological characteristics of rhythms will be considered under five headings: 1. persistence and initiation of rhythms, 2. phase control, 3. energy requirements, 4. period control and 5. entrainment.

1.4.1. Persistence and Initiation of Rhythms

Rhythms in a large number of biochemical, physiological and behavioural phenomena in a wide variety of organisms persist under certain specified constant environmental conditions, but may damp out, or actually be inhibited by other conditions. For example, the rhythm of sporulation in the alga *Oedogonium* persists in continuous light but is inhibited in constant darkness (Bühnemann, 1955a). The reverse is true however, of the green alga *Chlorella*; the rhythm of photosynthesis in this organism persists in darkness and low light intensities but not in continuous high light intensities (Hesse, 1972). Similarly, the rhythms of CO_2 output exhibited by leaves of *Bryophyllum fedtschenkoi* in a CO_2-free air stream (Wilkins, 1960a and b), and of luminescence in *Gonyaulax* continue in darkness and low light intensities but not in high light intensities (Haxo and Sweeney, 1955; Hastings and Sweeney, 1958).

The length of time for which a rhythm will persist under a particular set of constant conditions varies greatly from one organism to another. In general, long lasting rhythms are more common in animals than in plants; many activity cycles in rodents continue for several months in a uniform environment and a few rhythms have been found to persist even during hibernation (Menaker, 1961).

The eventual disappearance of a rhythm under constant environmental conditions may be due to one of the environmental parameters inhibiting the rhythm. If this is the case the rhythm usually disappears within a few hours and will begin again when the inhibiting environmental parameter is changed. On the other hand, the gradual damping out or disappearance of a rhythm in constant conditions may be due to one or both of two other phenomena. The first is desynchronisation of the rhythms in the individual cells of a population whether it is, for example, an algal culture or a multicellular plant organ like a leaf. The second is the gradual damping out of rhythms in the individual cells of the population so that all the cells are arrhythmic. These possibilities have to be considered in relation to re-initiation of rhythms by various stimuli because a

single stimulus could act to resynchronise the individual cells in the population as well as actually restart oscillation in each cell.

Attempts to distinguish between these two causes of the gradual damping out of circadian rhythmicity in organisms held in constant conditions have been made with unicellular organisms. The loss of rhythms in individual cells has been demonstrated in *Chlorella* (Hesse, 1972) and *Gonyaulax* (Sweeney, 1960) after prolonged exposure to constant conditions indicating that it is the damping out of rhythms in individual cells rather than desynchronisation which is involved. The exact reason for the gradual disappearance of rhythms in single cells has not yet been established.

In a case where a rhythm has been inhibited by prolonged exposure to a particular environmental parameter, then changing that parameter leads to the reinitiation of the rhythm. The rhythm of growth in *Avena sativa* coleoptiles is initiated by transferring seedlings from red light to darkness (Ball and Dyke, 1954, 1957) and the rhythm of CO_2 output in leaves of *Bryophyllum fedtschenkoi* kept in a CO_2- free air stream starts on transferring leaves from bright light into dim light or darkness (Wilkins, 1960a and b). The latter rhythm can also be initiated on transferring the leaves from an inhibitory temperature to one which permits oscillation (Wilkins, 1962b). In *Oedogonium* a transfer from continuous darkness to continuous light initiates the rhythm of sporulation (Buhnemann, 1955a) while the rhythm of petal movement in *Kalanchoë* can be initiated in response to both a "light on" and "light off" stimulus (Englemann,1960). In these cases the inhibiting environmental parameter appears to drive the oscillator to a fixed phase point from which it is released when the inhibiting environmental parameter is changed, and it is for this reason that the phase of the reinitiated rhythm bears a relationship to the time when the change in the environmental parameter occurs.

Rhythms which have gradually damped out in permissive conditions can be reinitiated by a single pulse-type change in an environmental parameter.

One of the most striking demonstrations of the initiation of a rhythm by such a stimulus is provided by the rhythm of eclosion in *Drosophila*. Cultures of *Drosophila* raised for several generations in continuous darkness show no rhythm in fly emergence. A single exposure to white light for 4 h, however, initiates a rhythm which continues in constant darkness for 10 - 14 days with a period of approximately 24 h (Pittendrigh, 1954).

In plants it is not always possible to apply this test because many are incapable of survival for long periods under constant conditions, especially in darkness. Nevertheless there are a few examples of the initiation of plant rhythms by a single stimulus. For example, a single change in light intensity will start the rhythm of luminescence in arrhythmic cultures of *Gonyaulax* previously maintained under continuous light for three years (Sweeney and Hastings, 1957). The rhythm of leaf movement in *Phaseolus* is initiated by a 1-h exposure to red light or to 5°C (Bünning, 1931) and, in the fungus *Pilobolus sphaerosporus* a rhythm of sporangium discharge can be initiated in response to a light flash of only 0.02 s duration (Bruce, Weight and Pittendrigh, 1960).

1.4.2. Phase Control

Knowledge of phase control is of crucial importance for two main reasons. Firstly, from a survival point of view it is an obvious advantage that endogenous rhythms operate in a definite phase relationship with external periodicities. Furthermore, in those multicellular organisms where a number of independent oscillators seem to operate their health and proficiency appear to be dependent on a specific phase relationship being established between the different oscillators. Secondly, investigations into the effects of a number of stimuli on the phase of a particular rhythm can provide valuable information about the nature of the underlying oscillator and its control.

The most important environmental parameters effective in resetting the phase of circadian oscillators are light, temperature and the partial pressure of oxygen.

The effects of anaerobic conditions on rhythms have however, been studied principally with the view of obtaining information about the energy requirements of the clock, and for this reason they will be discussed in the next section.

1.4.2.1. Phase control by light

In many organisms, a stimulus effective in initiating the rhythm also determines the phase. For example, the rhythms of growth rate in *Avena* coleoptiles and of CO_2 output in *Bryophyllum* leaves are inhibited by prolonged exposure to bright light. Transferring these organisms from continuous light (LL) to continuous darkness (DD) not only initiates the rhythms but also sets the phase which is determined by the time at which the transfer is made (Ball and Dyke, 1954, Wilkins, 1959, 1960a and b). Similar observations have been made for the rhythms of luminescence in *Gonyaulax* (Hastings and Sweeney, 1957) and of growth rate in the fungus *Neurospora crassa* (Pittendrigh, Bruce and Rosensweig and Rubin, 1959). On the other hand, the phase of the rhythms of leaf movement in *Phaseolus*, of sporulation in *Oedogonium*, and of CO_2 compensation in *Bryophyllum* is set on transferring these organisms from DD to LL (Bünning, 1965; Bühnemann, 1955a; Lörcher, 1958; Jones, 1973).

The influence of light on the phase of circadian rhythms persisting in darkness has been determined by investigations using a single light stimulus. Such investigations have revealed that the magnitude of the phase shift induced by a short light perturbation is dependent upon a number of factors including the position in the cycle at which the treatment is applied, the duration of the treatment, the radiant fluence rate, and the wavelength of radiation.

1.4.2.1.A. Position in the cycle

The effectiveness of a short light treatment in shifting the phase of a rhythm persisting in DD is highly dependent upon the position in the cycle at which the treatment is applied. In one part of the cycle a short light treatment may induce a large phase shift, whereas in another it may have little or no effect on the phase. For example, a 3-h light treatment will shift the phase of the luminescence rhythm in *Gonyaulax* if applied at a time in the cycle when minimum light production occurs. A similar light treatment applied when luminescence is at a maximum is, however, without effect (Hastings and Sweeney, 1958). Again, a phase shift can be induced in the rhythm of CO_2 output exhibited by leaves of *Bryophyllum fedtschenkoi* in darkness and CO_2 -free air by a single short exposure to light ending between the peaks of the rhythm while a similar treatment ending across a peak in the rhythm has no effect (Wilkins, 1960a and b; Harris and Wilkins, 1978b).

Scanning the whole circadian cycle with short light treatments enables the relationship between the magnitude of the phase shift and the position in the cycle at which the treatment is applied to be established. Such a relationship is known as a phase response curve. Detailed phase response curves have been obtained for rhythms in a wide variety of organisms. Those of CO_2 output in *Bryophyllum*, (Harris and Wilkins, 1978b), of luminescence and cell division in *Gonyaulax* (Hastings and Sweeney, 1958), of phototaxis in *Euglena* (Bruce and Pittendrigh, 1956) and of fly emergence in *Dropsophila* (Pittendrigh, 1960), are similar. Phase shifts are induced principally by light treatments which end in parts of the cycle which would normally occur at night, while little or no phase shift occurs in response to treatments ending in those parts of the cycle which normally occur during the day.

The phase shifting effects of dark pulses on rhythms persisting in LL have received far less attention. In *Gonyaulax* (Hastings and Sweeney, 1957) and *Acetabularia*, (Karakashian and Sweigner, 1976), *Bryophyllum* (Wilkins 1960a and

b). *Lemna* (Kondo, 1983) and *Euglena* (Malinowski, Laval-Martin and Edmunds, 1985), the phase response curves for light and dark pulses were found to be displaced by about 12 h along the time axis, indicating that the oscillator is sensitive to dark treatments in those parts of the cycle in which it is insensitive to light stimuli, and vice versa.

1.4.2.1.B. Duration of the treatment.

The magnitude of a phase shift induced by a light treatment applied at a particular position in the cycle is, in some cases, related to the duration of the treatment whilst in other cases this is not so. This depends upon whether or not the light treatment is a saturating one. A saturating light treatment is one which induces a phase shift that is not related to the duration of the treatment, but rather to some other property, such as the time at which it begins or ends. For example, in *Gonyaulax* the magnitude of the phase shift induced in the rhythm of luminescence by a light treatment applied 6 h after the organisms are placed in DD was found to be proportional to the duration of the treatment up to a maximum of 2.5 h (Hastings and Sweeney, 1958). A 2.5-h treatment induced a phase shift of 11 h, but longer exposures to light did not increase the magnitude of the shift. Thus in *Gonyaulax* a 2.5-h treatment can be described as saturating. In *Bryophyllum fedtschenkoi* maximum phase shifts in the rhythm of CO_2 output into a CO_2- free air stream are induced by light treatments ending between the peaks in the rhythm. Provided the treatment is of at least 3 h duration, the magnitude of the phase shift is not related to the length of the treatment but to the time at which it ends. A 1-h treatment ending at the same time as a 3-h treatment between the peaks of the rhythm induces a smaller phase shift (Wilkins, 1960 a and b; Harris and Wilkins, 1978b).

1.4.2.1.C. The radiant fluence rate

The effect of the radiant fluence rate (intensity) on the magnitude of a phase shift induced by a light treatment has received relatively little attention. Of the few studies which have been carried out it appears that in both plants and animals a relationship between these two variables exists only at low light intensities. In *Gonyaulax* for example, the phase shift induced by a short light treatment increases with increasing light intensity up to a maximum at 800 f.c. (Hastings and Sweeney, 1958). In *Bryophyllum fedtschenkoi*, the size of the phase shift is independent of the light intensity within the range of 8 - 3000 lux, but at lower light intensities there is evidence that the magnitude of the phase shift is related to the light intensity (Wilkins, 1960b). Similar findings have been reported for the rhythm of eclosion in *Drosophila* where the magnitude of the phase shift was found to be dependent upon the intensity of illumination up to a maximum of 10 lux (Chandrashekaran and Loher, 1969).

1.4.2.1.D. Wavelength of light

The effectiveness of a light treatment in inducing a phase shift is highly dependent on the wavelength of radiation employed. A number of so-called action spectra for phase shifting have been obtained, but in no organism has it yet been possible to identify unequivocally the photoreceptor system involved. What appears to be clear, however, is that different pigments are involved in different organisms.

In the algae, blue light appears to be the most active radiation in inducing phase shifts. Bühnemann (1955c) reported only blue light to be active in the *Oedogonium* sporulation rhythm. Blue light has recently been reported to shift the phase of the rhythm in chloroplast migration in single cells of *Acetabularia* which had previously been maintained in red light for several days (Schmid, 1986)

In *Gonyaulax* the action spectrum for phase shift induction has a major peak in the red with maximum effectiveness at 650 nm, and a minor peak in the blue region

of the spectrum with maximum effectiveness at 475 nm (Hastings and Sweeney, 1960). These authors suggest that a chlorophyll may be the photoreceptor molecule. In contrast, the action spectrum for the inhibition of luminescence is different from that for phase shifting in that activity in the red region extends into the near infra-red with a peak at 700 nm (Sweeney, Haxo and Hastings, 1959). Thus, there may be more than one pigment involved in the photo-control of the rhythm of luminescence in *Gonyaulax*. Ultraviolet (U.V.) light is also effective in inducing phase shifts in this organism (Sweeney, 1963), but the characteristics of the phase shifts induced differ from those induced by white light. Since they are known to absorb U.V. light, it has been suggested that nucleic acids mediate the U.V. light responses.

In common with most algae, photo-control of circadian rhythms in the fungi appears to be confined to the blue region of the spectrum. Whilst there have been a few reports of the relative effectiveness of different wavelenths of light in inhibiting rhythms in the fungi (e.g. Sargent and Briggs; 1967, Munzo and Butler, 1975), investigation has not been made of the spectral dependence of phase shifting in this group of organisms. The action spectrum for inhibition of the rhythm in conidiation in *Neurospora crassa* has a peak in the blue and a broad shoulder in the near ultraviolet spectral band (Sargent and Briggs, 1967). However, it cannot be assumed from this finding that blue light is necessarily the active component of the spectrum involved in phase shifting in this organism, particularly in view of the finding in *Gonyaulax* described above.

Several investigations in higher plants have indicated that the red and blue regions of the spectrum are most active in inducing phase shifts. The action spectrum for shifting the phase of the rhythm of CO_2 output in *Bryophyllum fedtschenkoi* shows activity only between 560 nm and 700 nm, with maximum activity at 660 nm (Wilkins, 1973). Whilst such a spectrum is indicative of phytochrome being the photoreceptor pigment, complete red/far-red reversibility

could not be demonstrated following a red light stimulus. Since the red light treatments were of 3 h duration, it is perhaps likely that the phase shifting process had gone beyond the photochemical stage before the far-red treatments were applied. Nevertheless, in rather complex entrainment experiments, the effects of repetitive 15 min, red light exposures were found to be completely nullified by an immediate subsequent exposure to far-red radiation for 15 min. This finding strongly indicates that phytochrome is the photoreceptor involved in phase shifting in *Bryophyllum*. (Harris and Wilkins, 1978a).

There is overwhelming evidence to indicate that phytochrome is involved in mediating phase shifts in the rhythm of leaf movement in *Samanae saman* (Simon, Satter, Galston, 1976). A 5-min exposure to red light shifts the phase of the rhythm, a response which can be abolished if the red light treatment is followed immediately by an exposure to far-red radiation. More recently, however, relatively long exposures (2 h) to blue light were also reported to shift the phase of this rhythm and the effects of this treatment were not abolished by a subsequent exposure to far-red radiation (Satter, Guggino, Lonergan and Galston, 1981). The characteristics of the phase shifts induced by blue light were similar to those induced by far-red radiation and led to the suggestion that in addition to phytochrome, a blue and far-red absorbing pigment may also be involved in mediating phase shifts in *Samanea*.

The phase of the rhythm of leaf movement in *Phaseolus vulgaris* can also be shifted by red, far-red and blue light (Lörcher, 1958). Whilst there is evidence to support the involvement of phytochrome in this organism, the possibility that a second blue and far-red absorbing pigment is also involved cannot be dismissed. An investigation into the photo-control of the rhythm of leaf movement in *Coleus blumeii* x *C. federici* led Halaban (1969) to conclude that more than one pigment may be involved in regulating the phase in this plant.

Space permits only a few studies to be mentioned here, but for further information the reader is referred to Harris (1977) where a more complete review

of the photo-control of circadian rhythms is provided.

1.4.1.E. Singularity point effects.

Brief exposure of organisms to light has been reported to inhibit the circadian system. This effect is phase dependent and was first reported in *Drosophila* (Winfree, 1970), where a light pulse of the correct dose given at a specific phase point in the cycle, now called a singularity point, could abolish the rhythm of eclosion. Singularity points have since been reported in *Kalanchoë* (Englemann, Johnsson, Karlssson, Kobler and Schimmel, 1978), *Lemna* (Kondo,1983) and more recently in *Euglena* (Malinowski *et al.*, 1985), and their occurrence has led to the suggestion that biological oscillators may belong to a group of oscillators showing limit cycle dynamics.

1.4.2.2. Phase Control By Temperature

There are two ways in which temperature can influence the phase of circadian rhythms. Firstly, temperature may set the phase in organisms transferred from an inhibitory temperature to one which permits oscillation. Secondly, a brief change in temperature may modify the phase of an already established rhythm.

Very few attempts have been made to study the nature of the inhibition of a rhythm in organisms held at extreme temperatures by transferring them to temperatures which permit oscillation. Of the few investigations which have been made, it would appear that during prolonged exposures to inhibitory temperatures the basic oscillator is held at a fixed phase point from which it is released when the temperature is restored to a value within the functional range. The rhythm of CO_2 output exhibited by leaves of *Bryophyllum fedtschenkoi* kept in a CO_2- free air stream and darkness, for example, is inhibited if the leaves are exposed to 36^oC. Transferring the leaves to 16^oC initiates the rhythm and the phase is set by the

time at which the transfer takes place (Wilkins, 1962b). Transferring organisms from a low temperature to a higher one may also restart rhythms and set their phase. The rhythm of leaf movement in *Phaseolus* is inhibited at 5°C, but begins on increasing the temperature to 20°C. The first peak of the rhythm always occurs a definite time after the end of the low temperature treatment regardless of the time at which the temperature change is made (Bünning, 1961). Similar findings have been reported for the rhythm of luminescence in *Gonyaulax* (Hastings and Sweeney, 1957), of mating in *Paramecium* (Ehert, 1959) and that of K^+ uptake in *Lemna gibba* G3 (Kondo Tsudzuki, 1980).

The phase of a rhythm which is set by a change from light to darkness is, in some species, also influenced by temperature. In *Kalanchoë* and *Bryophyllum* the time taken to reach the first peak of the rhythm after a transfer from light to darkness increases with decreasing temperature (Schmitz, 1951; Wilkins, 1962b). No such effect was observed for the rhythm of stomatal opening in *Xanthium pennsylvanicum* where the first peak of the rhythm occurs after 16 h within the range of 15 to 36°C (Mansfield and Heath, 1964).

In contrast to the relatively few studies which have been carried out on the effects of prolonged exposures to extreme temperatures on rhythms, there are numerous reports of the effects of pulse-type temperature stimuli on the phase of circadian rhythms.

The effectiveness of short-duration temperature changes in shifting the phase of a rhythm depends upon the position in the cycle at which the treatment is applied, the duration of the treatment, whether the temperature is increased or decreased and the magnitude of the change.

A detailed study of the effects of short exposures to high (36°C) temperature has been made on the rhythm of CO_2 output exhibited by leaves of *Bryophyllum* kept in darkness and a CO_2-free air stream (Wilkins, 1983). In this plant the effect of a high temperature treatment is virtually identical to that of a short exposure to

light, the similarity extending to the position in the cycle at which a treatment is effective, as well as the magnitude and direction of the phase shift induced. High temperature resets the phase when the treatment is applied between the peaks but not at the tops of the peaks of CO_2 output and, as with a light stimulus, provided the temperature treatment is at least 3 h in length, the magnitude of the phase shift is not related to the duration of the treatment but rather to the time at which it ends (Wilkins, 1962b,). The phase response curve for high temperature in *Bryophyllum* is similar to that reported by Moser (1962) for *Phaseolus multiflorus*. Increasing the temperature from 20 to 28°C for 4 h induced a phase shift in the rhythm of leaf movement provided the treatment was given during the night phase of the rhythm when the leaves were in a downward position. The phase was delayed by treatments ending in the first half of the night phase, and advanced by treatments ending in the second half of the night phase. Treatments ending in the day phase of the rhythm, when the leaves were in the up position, had little or no effect on the phase. As with *Bryophyllum*, the phase reponse curve for high temperature in *Phaseolus* is virtually identical to that for light. The equivalence of high temperature and light pulses have also been reported for phase-shifting in the rhythm of K^+ uptake in *Lemna gibba* G3 (Kondo, 1983).

Low temperature pulses are also effective in inducing phase shifts in the rhythms of a number of organisms. A detailed phase response curve for low temperature has been published by Wagner (1963) for the rhythm of leaf movement in *Phaseolus multiflorus*. Decreasing the temperature from 20°C to 4.5°C for 4 h at various points in the cycle indicated that low temperature was effective in inducing phase shifts in those parts of the cycle where high temperature had previously been found to be without effect (Moser, 1962) and vice versa. A somewhat similar situation may occur with the rhythm of CO_2 output in leaves of *Bryophyllum fedtschenkoi* kept in a CO_2-free air stream and darkness (Wilkins, 1962b). A detailed study of the effects of low temperature in this organism was not

made, but the results of chilling the leaves at a few positions in the cycle indicated that low temperature was effective in inducing phase shifts in those parts of the cycle where high temperature was not. In contrast to high temperatures, which can cause both delays and advances in the phase, low temperatures were found only to delay the phase in both *Phaseolus* and *Bryophyllum* (Wagner, 1963; Wilkins, 1962b). Since the effects of low temperatures on the *Bryophyllum* rhythm were investigated at only a few positions in the cycle, it is possible that phase advances may be induced in other positions. Low temperatures also appear to be ineffective in inducing phase advances in other organisms such as *Oedogonium cardiacum* (Ruddat, 1961) and *Avena sativa* (Ball and Dyke, 1957), where rhythmic sensitivity to such stimuli have been reported. However, in a recent study in *Lemna gibba* G3 (Konda, 1983) decreasing the temperature from 26°C to 5°C was found to be effective in both advancing and delaying the phase of the K^+ uptake rhythm. The magnitude and direction of the phase shift was found to be dependent on the precise position in the cycle at which the treatment was applied. Moreover, in this organism the effects of a low temperature pulse were virtually identical to that of a dark pulse with respect to the position in the cycle at which the treatments were effective, and the magnitude and the direction of the phase shift induced. The equivalence of low temperatures and darkness in inducing phase shifts has also been reported for the rhythm of sporulation in *Oedogonium* (Bünning and Ruddat, 1960), of CO_2 output in *Bryophyllum fedtschenkoi* (Wilkins, 1960a and b, 1962b) and, more recently, for stem extension in *Chenopodium rubrum* (Lecharny *et al.*, 1985).

Thus, in general it appears that the basic circadian oscillator is sensitive to low temperatures and darkness in those positions in the cycle at which it is insensitive to high temperatures and light, and vice versa.

There have been few investigations made of the relationship between the magnitude of a phase shift induced and, a) the duration of a temperature treatment and, b) the magnitude of the temperature change. In *Bryophyllum fedtschenkoi*,

the magnitude of the phase shift induced in the rhythm of CO_2 ouput by a high temperature treatment is not related to the duration of the treatment but only to the time at which it ends, provided the treatment lasts for at least 3 h. A 1-h high temperature treatment may give rise to a smaller phase shift, although this depends upon the point of application in the cycle. The results of exposing the leaves to low temperatures for various durations were however more complex. At certain positions in the cycle low temperatures gave rise to phase shifts which approximately equalled the duration of the treatment. Similar findings have been reported by Ball and Dyke (1957) for the rhythm of growth rate in *Avena* coleoptiles.

In *Bryophyllum* phase shifts were also induced by exposing leaves to an increase or decrease in temperature within the functional range, the magnitude of the phase shift induced by such treatments was however, smaller than that induced by inhibitory temperatures applied at similar positions in the cycle (Wilkins, 1962b).

1.4.3. Energy Requirements of the Clock

Investigation of the energy requirements of the basic circadian oscillator has involved exposing organisms to anaerobic conditions for different lengths of time and at various positions in the cycle. The results of these experiments have often been compared with similar experiments using low temperatures, since lack of oxygen and low temperatures may be expected to reduce aerobic metabolism to a low level.

The effect of anaerobic conditions on various rhythms has been studied in several organisms by replacing the air supply with oxygen-free nitrogen. Several such studies have indicated that anaerobiosis delays the phase of circadian rhythms. In *Avena* , for example, the delay in the phase of the rhythm in

coleoptile growth rate was found to be equal to the duration of the treatment (Ball and Dyke, 1967). However, the delay on the second peak was much less, indicating that the oscillator may have been speeded up. Similar results were found on exposing coleoptiles to low temperatures. These experiments also revealed that there was no point in the cycle at which the oscillator was insensitive to the anaerobic treatment. In contrast, this was not found to be so for the rhythms of petal movement in *Kalanchoe blossfeldiana* , of leaf movement in *Phaseolus multiflorus* (Bünning, Karrus and Vielhaben, 1965), or of CO_2 output in *Bryophyllum fedtschenkoi* (Wilkins, 1967). In each of these organisms, there were specific positions in the circadian cycle where insensitivity to anaerobic conditions was observed. In *Bryophyllum fedtschenkoi*, a permanent phase shift was induced when the leaves were exposed to anaerobic conditions for 6 h between the peaks of CO_2 output (Wilkins, 1967). The delay on the first peak was approximately equal to the duration of the treatment, but that on the second and third peaks, whilst always being closely similar, was usually slightly less than the delay on the first peak. This finding indicated that the period of the rhythm may have gained stability between the first and second post-treatment peaks. Withdrawing O_2 for 6 h near the top of a peak in the rhythm did not induce a phase shift, but when the treatment lasted for more than 8 h a phase shift was induced, the magnitude of which was approximately half the duration of the treatment.

The fact that some organisms show periodic sensitivity to anaerobic conditions and low temperatures led Bünning (1960) to propose that the basic circadian oscillator is a relaxation-type oscillator, in which a tension phase requiring metabolic energy alternates with a relaxation phase in which energy is not required. But two facts appear to argue against this being the case. Firstly, some organisms are sensitive to anaerobic conditions in all parts of the cycle and secondly, Bünning himself later discovered that in *Phaseolus* the positions in the cycle at which the oscillator is sensitive to anaerobic conditions and low

temperatures do not coincide exactly (Bünning, 1965).

1.4.4. Period Control

The control of the period is of critical importance in an oscillating system which forms the basis of time measurement. The free-running periods of most circadian rhythms lie within the approximate range of 20 - 28 h. The length of the period in a particular organism is not absolutely fixed, but may vary slightly in response to changes in environmental conditions. The most important environmental parameters which influence the length of the free-running period are light and temperature.

1.4.4.1. The effects of light

Several investigations of plant rhythms have indicated that the length of the free-running period is dependent upon the radiant fluence rate and the spectral composition of radiation to which the organism is exposed.

In *Gonyaulax*, the period of the rhythm in luminescence decreased from 24.5 h at 120 f.c to 22 h at 680 f.c. (Hastings and Sweeney, 1958). Similar findings have been reported by Bünsow (1953) for the rhythm in petal movement in *Kalanchoë blossfeldiana* and more recently by Buchanan-Bollig (1984) for the rhythm of CO_2 exchange exhibited by leaves of *Kalanchoë daigremontiana*.

Harris and Wilkins (1976) made a detailed investigation of the effects of varying the radiant fluence rate and the spectral composition on the rhythm of CO_2 output exhibited by leaves of *Bryophyllum fedtschenkoi* kept in a CO_2-free air stream at 15°C. They reported that the period of the rhythm in the light, 22.3 h, was significantly shorter than that of 23.7 h recorded in leaves maintained in continuous darkness. A significant reduction in the period occurred in response to monochromatic radiation centred on 530 nm, 600 nm, 660 nm, and 730 nm. The most effective band, however, was centred on 660 nm. In further study using this band

the period was found to decrease as the quantum fluence rate increased.

Light quality has also been reported to modify the length of the period of leaf movement in *Phaseolus multiflorus* (Lörcher, 1958) and *Coleus* (Halaban, 1969). In *Phaseolus* the period lengthened in red-light but shortened in far-red radiation in comparison with the value in darkness. In *Coleus*, however, the period in red light, 20.5 h, was significantly shorter than that of 22.5 h recorded in leaves maintained in darkness. Blue light lengthened the period in this plant, whilst green and far-red were without effect.

The free-running period in a large number of animals reacts to light intensity in a predictable manner which has become known as Aschoff's rule (Aschoff, 1965). This rule states that with increasing light intensity, the period in nocturnal animals decreases whilst that in diurnal animals increases. There are, however, a few exceptions to this rule, notably among primates.

1.4.4.2. The effects of temperature

The single, most important, feature which enables circadian oscillators to provide the basis for accurate time measurement is their ability to oscillate with a reasonably uniform frequency despite changes in ambient temperature. As already pointed out, the period of circadian rhythms is not independent of temperature, but changes slightly with increasing or decreasing temperature. Hastings and Sweeney (1960) have provided a comprehensive list of period lengths in organisms held at different temperatures, from unicellular plants to mammals. These data show that the temperature coefficients of most circadian systems are rarely greater than 1.2, and usually lie within the range of 1 - 1.1.

In most organisms the free-running period shows a slight decrease with increasing temperature. There are, however, a number of exceptions. The periods of the rhythms of leaf movement in *Phaseolus multiflorus* (Leinweber, 1956) and of growth rate in *Avena sativa* (Ball and Dyke, 1954) show almost complete temperature independence between approximately 15^{0}C and 25 - 28^{0}C. In three

other organisms, the period shows a slight increase as the temperature is raised. This response was first reported by Bühnemann (1955b) for the rhythm of sporulation in *Oedogonium*, and was subsequently found to occur in the rhythm of luminescence in *Gonyaulax* (Hastings and Sweeney, 1957) and in running activity in the cockroach (Bünning, 1958).

The mere fact that temperature does influence the length of the free-running period is good evidence for the endogenous nature of the oscillator, and clearly implies that it must incorporate some form of temperature compensating process. The mechanism of temperature compensation is unknown, but it has been proposed that a mechanism in the oscillating system itself is responsible for slowing down some processes to compensate for the speeding up of others with the result that very little over all change occurs (Bünning, 1973).

1.4.5. Entrainment

Under natural conditions the period of circadian rhythms is entrained to precisely 24 h. Entrainment to 24 h periods can be achieved by alternating periods of light and dark, or subjecting organisms to temperature fluctuations.

A substantial contribution to the understanding of the environmental control of rhythms by light-dark cycles was made by Kleinhoonte, who in 1929, found that the rhythm of leaf movement in *Canavalia ensiformis* could be regulated to a period of precisely 24 h by light breaks of only 1 min duration every 24 h. Further studies revealed that the minimum exposure of light required to entrain a rhythm to a 24-h period was to some extent dependent upon the organism under investigation. A 15-min exposure to light in every 24 h cycle is sufficient to entrain the rhythm of CO_2 output in *Bryophyllum* (Harris and Wilkins 1978b). Similar results have been reported in *Lemna perpusilla* (Hillman, 1964) and in *Drosophila* (Pittendrigh and Minis, 1964). However, in order to entrain the rhythm of leaf movement in *Pharbitis nil*, 1 - 4 h light in each 24-h cycle is required

(Bollig, 1974).

Rhythms may also be entrained to periods other than 24 h by light-dark cycles. The period of the rhythm in leaf movement in *Canavalia ensiformis* can be entrained to 16 or 18 h by light-dark cycles of 8:*8* or 9:*9* h respectively (Kleinhoonte, 1929). However, when exposed to a 6:*6*-h cycle no entrainment occurs, the period remaining at approximately 24 h. The rhythm of luminescence in *Gonyaulax* can be entrained by 6:*6*, 7:*7*, 8:*8* and 16:*16*-h cycles of light and dark (Hastings and Sweeney, 1958). In *Oedogonium* entrainment may occur with 9:*9*-h cycles but not with 6:*6*, 15:*15* or 24:*24*-h cycles (Bühnemann, 1955c). These findings have led to the conclusion that the limits of entrainment are a fixed property of the oscillating system. However, evidence against this being the case was provided by Wilkins (1962a) who reported that the limits to which the rhythm of CO_2 output in *Bryophyllum* could be entrained were a function of the light intensity. At 1000 lux the rhythm could be entrained by 3:*3*, 6:*6* and 8:*8*-h cycles of light and dark, at 500 lux entrainment by 3:*3*-h cycles was not possible and at 100 lux no entrainment occurred in response to 6:*6*-h cycles. Similar findings have been reported for the rhythm of luminescence in *Gonyaulax* (Hastings and Sweeney, 1958). With the exception of the alga *Hydrodictyon reticulatum* (Pirson, Shön and Doring, 1954; Schön, 1955), organisms show no tendency to retain the imposed frequency of the entraining cycle when placed under constant conditions, but revert immediately to the normal free-running period.

Temperature cycles are also effective in entraining the period of circadian rhythms. The relatively few studies that have been carried out have been summarised by Sweeney and Hastings (1960). The entraining effect of temperature cycles was first reported by Stern and Bünning (1929) who found that they could reverse the phase of the rhythm of leaf movement in *Phaseolus* by having the maximum temperature at night. Bühnemann (1955b) showed that the sporulation rhythm in *Oedogonium* could be entrained by a day and night temperature differential of only 2.5°C and in *Kalanchoë*, a day-night temperature differential

of only 1°C has been reported to entrain the rhythm of petal movement (Oltmans, 1960). Entrainment to non-24-h periods is also possible with temperature cycles. Bühnemann (1955c) demonstrated that a 9:*9*-h cycle of different temperatures could entrain the period of sporulation in *Oedogonium* to 18 h but a 15:*15*-h cycle was ineffective .

In a study of the interaction of light and temperature cycles in *Drosophila* , Pittendrigh (1958) concluded that light entrains more strongly than temperature. The same conclusion was reached with studies carried out in *Euglena* (Bruce, 1960) and *Periplaneta americana* (Roberts, 1960). Wilkins (1965) has expressed reservations about such a conclusion since the majority of studies have failed to take into consideration the magnitude of the temperature fluctuation and the intensity of light used in the entraining cycle.

1.5. LOCATION AND IDENTIFICATION OF THE BASIC OSCILLATOR

The ultimate aim of research in the field of circadian rhythmicity is to locate and identify the underlying oscillator. This aspect of the subject has constituted the most active area of research in recent years. A few studies have addressed the question of whether organisms have one or several oscillators. The persistence of rhythms in individual cells of *Chlorella* (Hesse, 1972) and *Gonyaulax* (Sweeney, 1960) clearly demonstrates that each cell must contain its own circadian pacemaker. Several investigations in multicellular organisms have indicated that the level of organisation at which oscillation operates depends upon the organism in question. For example, the rhythm of CO_2 output exhibited by leaves of *Bryophyllum fedtschenkoi* maintained in darkness and a CO_2-free air stream has also been detected in pieces of mesophyll tissue excised from any part of the leaf from which the epidermis has been removed (Wilkins, 1959) and also in unorganised callus

tissue from leaves of *Bryophyllum daigremontiana* (Wilkins and Holowinsky, 1965) suggesting that in these plants each cell contains its own pacemaker. In *Phaseolus*, however, the periodic changes in the turgidity of the pulvinus cortical cells which give rise to the rhythm of leaf movement are dependent on the presence of the lamina (Brauner and Arslan, 1951). This is not the case, however, for the rhythm in leaf movement in *Samanea saman* (Palmer and Asprey, 1958). Rhythms in the turgidity of the cortical cells of the pulvinus in this plant have been found to continue even when the extensor and flexor regions of the pulvinus are isolated.

In organisms which show more than one rhythm, the possible existence of several basic oscillators, each responsible for a particular rhythm or group of rhythms, can be investigated. If each overt rhythm is controlled by a separate oscillator then some degree of dissociation between the individual rhythms may occur in response to external stimuli, whereas if a common oscillator is responsible for all the rhythms they should retain a definite phase relationship with one another. All three rhythms which have been recorded in *Gonyaulax* , i.e. luminescence, cell division and photosynthesis, have the same period, and the phase of each is shifted to the same extent in response to external perturbations (McMurray and Hastings, 1972). Whilst these findings indicate that one oscillator underlies all three rhythms, the possibility that three basic oscillators exist, each having the same period and response to external stimuli cannot be eliminated. In man, an artificial light-dark cycle can entrain the body temperature rhythm immediately, whilst the K^+ secretion rhythm takes 8 - 9 days to entrain. (Loban, 1965). Moreover, the free-running periods of the body temperature rhythm and the K^+ secretion rhythm differ, suggesting that these rhythms may be controlled by two distinct oscillators (Aschoff, Grerecke and Wever, 1967).

The remarkable similarity between the physiological characteristics of rhythms in organisms as diverse as unicellular plants and complex multicellular organisms such as man, has led to the idea that a basic oscillator, common to all organisms, underlies the generation of these rhythms. A number of models for this

oscillator have been proposed. These models can be divided into two groups; mathematical models, and models which are based on structural, physiological and biochemical components common to all eukaryote organisms. Mathematical models are based on the assumption that the circadian oscillator follows simple mathematical rules. No attempt will be made here to give a description of these models but the reader is referred to Weaver (1965) and Pavlidis (1973), where detailed information can be found. In brief, a number of equations have been formulated to explain the properties of biological oscillators, but none gives any clue as to the biochemical or molecular nature of the oscillator and, as pointed out by Hastings and Keynan (1965), "for an ultimate understanding of these systems we must turn to the genetic and molecular level; inevitably we are seeking the solution to the question of mechanism in terms of chemical activities and transformations".

As previously mentioned, a number of physiological models for the basic oscillator have been proposed; reference will be made to two of these, the "Chronon Concept" of Ehert and Trucco (1969) and the membrane model of Njus *et al.* (1974). It must be emphasized, however, that there is as yet no unequivocal evidence that the basic oscillator is the same in all organisms. Many investigators, particularly within the last few years, have sought an understanding of the generation of a particular rhythm in a particular organism rather than formulating models which apply to all organisms.

Since space is limiting, this section will be confined almost entirely to reviewing the relatively few investigations which have been made of the mechanisms underlying rhythms in plants, and the fungus *Neurospora crassa.* Reference will however be made to animal systems where appropriate.

Two main approaches have been taken with the view of establishing the biochemical and molecular events underlying circadian rhythms. In the first, attempts have been made to isolate the basic oscillating system either by surgically removing organelles, or by studying "clock" mutants in which the mutation has

resulted in a particular aspect of a rhythm being disrupted. The second approach has been either to trace back a series of biochemical pathways from the observed rhythm to the underlying oscillator or, to examine the effects of a number of metabolic inhibitors on the period and phase of a rhythm. Some investigations have involved only one of these approaches whilst others have involved a combination of the two.

The vital role played by nucleic acids and proteins in the function of the cell led to the proposal that the patterns or rates of synthesis of nucleic acids or proteins may be the primary oscillating mechanism. Such a view was advanced by Ehert and Trucco (1969) in the "Chronon Concept". This model proposes that circadian rhythms arise from rhythmic transcription of a large polycistronic complex of DNA, the chronon, the transcription rate of which is governed by some function of eukaryote cells which is relatively temperature independent. Chronons are assumed to be contained on each of the nuclear chromosomes, in addition to being present in other organelles. It is proposed that transcription proceeds unidirectionally along the chronon, each cistron coding sequentially for an enzyme which occurs in a similar 24-h sequence. The last chronon, however, codes for an initiator substance which diffuses back to the initiator cistron and the system proceeds to its next circadian cycle.

This model clearly places the basic oscillator or the biological clock at a molecular level. Although there is no substantial experimental evidence to support it, it will become evident in this section that some rhythms may well be generated and controlled at the level of transcription.

The role of protein and nucleic acid synthesis in the generation of circadian rhythms has been investigated in *Acetabularia*. The very first experiments were carried out by Sweeney and Haxo (1961) in an attempt to establish the part played by the nucleus. Surgical removal of this organelle was found to have no effect on the rhythm of photosynthetic capacity, which continued in enucleate cells for several weeks under constant conditions. Moreover, the phase of the rhythm in

enucleate cells could be set by external cues in a manner identical to that found in intact cells. Whilst these results clearly demonstrated that the rhythm of photosynthesis was not dependent upon the presence of the nucleus, this study did not establish whether the nucleus also had a circadian oscillator. This question was investigated by Schweiger and Schweiger (1965) who interchanged the nuclei between cells which had been entrained by light-dark cycles so that their rhythms were 180^0 out of phase. When these cells were transferred to constant conditions the phase of the rhythms was found to correspond to the light-dark cycle to which the nucleus had been exposed. The nuclei must therefore contain a master clock which has a dominant role in determining the phase.

The continuation of the rhythm in enucleate *Acetabularia* cells did not rule out the involvement of nucleic acid and protein synthesis in the oscillating mechanism because these cells contain large amounts of DNA in their chloroplasts (Gibor and Izawa, 1963) which can direct the synthesis of all the essential proteins (Schweiger and Berger, 1961; Schweiger and Bermer, 1964). Investigations aimed at establishing the role of nucleic acids and protein synthesis in the oscillating mechanism of *Acetabularia* have involved the use of inhibitors. High concentrations of chloramphenicol, actinomycin D and puromycin were found to be without effect on the phase and period of the rhythm of photosynthetic capacity, but all these inhibitors caused about a 50% inhibition of ^{14}C leucine incorporation (Sweeney, Tuffli, Rubin, 1967). At first sight these results may suggest that protein and nucleic acid synthesis were not involved in the central oscillating mechanism in *Acetabularia* . However, it was later found that a large fraction of RNA metabolism in this organism is unaffected by actinomycin D (Sweeney *et al.*, 1967), and it is possible that only a small fraction of the total RNA is involved in the clock. Recently, a polypeptide, p230 has been isolated from the chloroplast fraction of this organism (Hartwig, Schweiger and Schweiger, 1985). The amount of this protein oscillates with a period of about 24 h under constant conditions and shows a clear phase relationship with the photosynthesis rhythm. Furthermore, the phase and

period of the rhythm in p230 synthesis is affected by cycloheximide, an inhibitor of translation on the 80S ribosomes, whereas chloramphenicol, which inhibits translation on the 70S ribosomes has no effect. These findings therefore suggest, that the p230 protein is translated on the 80S ribosomes and may be involved in the central mechanism of the clock in *Acetabularia*. Whether or not a rhythm also occurs at the transcription level was not established but such information would clearly be of value in assessing whether or not the results in this study can be interpreted in terms of the "Chronon Concept".

The possible role of protein synthesis in the mechanism underlying the rhythm of luminescence in *Gonyaulax* has also been investigated. Early studies showed that this rhythm was due to periodic variation in the level of the enzyme and subtrate involved in the reaction. Luciferase has been shown to vary diurnally (Hastings and Keynan, 1965), and a daily rhythm in the level of the substrate luceferin has also been reported (Bode, DeSa and Hastings, 1963). The possibility that the rhythm in luciferase level was a result of periodic synthesis of the messenger RNA coding for this enzyme was investigated by studying the effects of various inhibitors on the rhythm. (Karakashian and Hastings, 1963). Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, abolished the luminescence rhythm. The inhibition of the rhythm was not, however, immediate, one peak of luminescence was observed following the exposure to actinomycin D, suggesting that the fraction of RNA responsible for a peak in luminescence is synthesized 24 h earlier. Puromycin, an inhibitor of translation on the 70S and 80S ribosomes abolished the rhythm immediately as would be expected since in effect the assembly of the protein would be arrested. However, chloramphenicol had no effect on the phase or period of the rhythm, but enhanced the amplitude, while mitomycin, an inhibitor of DNA synthesis, had no effect on the rhythm until 3 days after application. It has been suggested that the effects of the latter may be explained by a slow rate of turnover of the fraction of DNA involved in the clock. It is equally possible, however, that mitomycin did not actually penetrate the cells, a point

which Karakashian and Hastings (1963) do not appear to have considered.

In order to demonstrate that an inhibitory substance actually affects the underlying oscillator it is necessary to show that brief exposure to such a substance results in either a phase shift or change in period length. A major practical difficulty with this approach however, is that it is not always possible to remove completely the inhibitor from the cells at the end of the treatment.

Pulse-type treatments of actinomycin D were found to inhibit the luminescence rhythm in *Gonyaulax* in the same way as prolonged treatments (Karakashian and Hastings, 1963), and a 3-h treatment with DCMU, which totally inhibits photosynthesis in this organism, had no effect on the phase and period of the rhythms in photosynthetic capacity or luminescence (Sweeney, 1969). These results did not, therefore, enable the role of protein synthesis in the *Gonyaulax* clock to be established. A number of more recent studies have, however, indicated that protein synthesis may be an essential part of the basic oscillator in this organism. Waltz *et al.* (1983) have provided the first true evidence for a rhythm in RNA content, which may underlie the rhythm of total protein content reported by several other investigators (e.g. Cornelius, Schroeder-Lorenz and Rensing, 1985; Volknandt and Hardeland, 1984). This rhythm persisted in continuous light with a period of approximately 23 h, closely similar to that of the rhythm in luminescence measured under the same conditions. Maximum RNA content appeared at a time in the cycle when maximum sensitivity to light and cycloheximide were observed and, moreover, specific new RNAs appeared at this time and disappeared 3 - 4 h later when a steep decline in RNA content was observed. Furthermore, short, 1-h pulses of anisomycin, streptimidone (Taylor, Dunlap and Hastings, 1982) and cycloheximide (Dunlap, Taylor and Hastings, 1980), inhibitors of protein synthesis on the 80S ribosomes, were all found to be effective in shifting the phase of the luminescence rhythm, the magnitude and direction of the shift depending upon the time in the cycle at which the inhibitor was applied. This finding indicated that, as in *Acetabularia*, 80S ribosome protein synthesis may be of key importance in the

generation of the luminescence rhythm in *Gonyaulax*.

The circadian oscillator in *Neurospora crassa* has also been investigated at the molecular level. In continuous darkness and at a constant temperature, this organism exhibits a circadian rhythm in conidiation with a period of about 22 h (Sargent and Briggs, 1967). "Clock" mutants with respect to three aspects of the rhythm have been isolated, and recently reviewed by Feldman (1982). For example, the period is altered by mutations at several loci (Feldman and Dunlap, 1983; Feldman and Hoyle, 1973). A maternally inherited gene, *poky*, can affect the sensitivity of the rhythm to light (Brian, Freeberg, Weiss and Briggs, 1977), and a mutation, *cel*, which disrupts the structure of the fatty acid synthetase results in partial loss of temperature compensation below 22°C, suggesting that fatty acid metabolism may be involved in temperature compensation (Elovson, 1975). The most recent mutant to be reported, frq^{-9}, shows complete loss of temperature compensation (Loros, Richman and Feldman, 1986). A number of oligomycin-resistant (oli^{r}) mutants have also been isolated having periods 2 - 3 h shorter than the normal strains, and in several, the amino acid composition of the DCCD-binding protein found in the F_0 portion of the mitochondrial ATP synthetase complex differs from that of the normal strain (Brody, Dieckmann and Mikolajczyk, 1985). These authors point out that such a finding does not necessarily indicate that the ATP synthetase complex is part of the *Neurospora* oscillator and suggest that alterations to the ATP synthetase complex may disrupt the proton gradient across the mitochondrial membrane.

Mutations which alter the period of rhythms have also been reported in *Chlamydomonas* (Bruce, 1972) and *Drosophila* (Konopka and Benzer, 1971). Recent investigations in *Drosophila* have provided convincing evidence for the genetic nature of the oscillating system. A mutation at a single gene locus, the *per* gene, affects the period of the rhythm in eclosion, and that of the 1-minute cycle of the courtship song, the periods becoming either longer or shorter, or rhythmicity being entirely abolished. The *per* gene has now been cloned (Bargiello and

Young, 1984), and cloned segments have the ability to restore rhythmicity either partially or fully, when introduced into arrhythmic mutants (Zehring, Wheeler, Reddy, Kyriacou, Rosbasch and Hall 1984; Bargiello, Jackson and Young, 1984). Moreover, the *per* gene has been found to contain an unusual DNA sequence, homologous to the DNA of chickens, mice and humans (Shin, Bargiello, Clark, Jackson and Young, 1985). Whether or not this DNA is part of the clock in these organisms is, however, uncertain.

The majority of investigations into the role of protein and nucleic acid synthesis in biological clock mechanisms has involved either the use of inhibitors or genetic mutants. The results obtained from both types of experiment must be interpreted with caution. The specificity of many inhibitors is often uncertain and there may be secondary effects. Similarly, genetic manipulations may not act directly on the basic oscillator but indirectly by altering a cellular process which impinges upon a component of the underlying oscillator.

In no organism has it been possible to provide unequivocal evidence that the synthesis of proteins and nucleic acids form an integral part of the basic oscillator, and so a number of other proposals have been made.

The similar structure and function of membranes in all eukaryotes, together with several observations that a number of chemicals known to affect membranes disrupt circadian systems, led Njus *et al.* (1974) to propose a membrane model for circadian oscillators. This model, which is based on the fluid mosaic model for membrane structure (Singer and Nicolson, 1972), proposes that the distribution of protein particles in the lipid membrane change in response to changing ion concentrations, aggregating to form channels when the gradients are low and dispersing to block transport when the gradients are high. These changes operate in a concentration-dependent, feedback mechanism with the result that a slow cyclical change in the ion concentration on the two sides of the membrane occurs. The kinetics of the oscillation are assumed to depend upon the activation and inhibition of the membrane proteins, the kinetics of the transport process and the

fluidity of the membrane lipids. Temperature compensation of the circadian period is thought to be a result of lipid adaptation which is relatively temperature independent (Baránska, and Wlodawer, 1969)

The model also accounts for the phase shifting effects of light by proposing that light sensitive ion "gates" in the membrane open in response to light thus lowering or abolishing the ion gradients. A light treatment applied when the membrane is in an active state will cause a phase delay since the membrane will need to repeat the accumulation of ions until maximum concentration is reached. A phase advance would result from a light treatment applied when the membrane is in the passive state since it would reduce the time necessary for the disappearance of the ion gradient. The induction of phase shifts by temperature stimuli are explained by lipid adaptation taking several hours to complete and during this time the ion gradients across the membranes become disrupted. Evidence that lipid adaptiaton does in fact take several hours has come from investigations in *E. coli* (Okuyama, 1969) and earthworms (Rao, 1967).

There are a number of circadian systems which can clearly be attributed to the movement of ions, particularly those which control leaf movement. The role of ion transport in the control of leaf movement in a number of plants was reviewed by Satter and Galston in 1981. Ion fluxes undoubtedly underlie the rhythmic turgor changes in the cortical cells of the pulvinus which give rise to the rhythm of leaf movement in a number of plants including *Samanea saman*, *Phaseolus multiflorus*, and *P. vulgaris*, *Albizzia julibrissin* and *Mimosa pudica*. In *Samanea saman*, for example, the rhythm has been attributed to an alternating predominance of inwardly directed ion pumps and outward leakage via diffusion channels in the plasmalemma of the motor cells in the extensor region of the pulvinus. White light appears to shift the phase of this rhythm by activating outwardly directed K^+ pumps (Satter, Geballe, Applewhite and Galston, 1974).

Whilst K^+ is a major cation controlling rhythmic leaf movement, other ions are clearly involved. Chloride is of major importance in balancing positive charges

but other anions participate. Organic ions are known to be important in neutralising positive charges in guard cells (Allaway, 1977; Snaith and Mansfield, 1985). Recently, Bialczky and Lechowski (1986) have reported diurnal changes in the concentration of malic acid in the pulvini of *Phaseolus coccineus*, and believe that malic acid has the same function as it does in guard cells.

Snaith and Mansfield (1986) have recently provided evidence for the involvement of K^+ and Cl^- fluxes in the circadian rhythm of stomatal opening in *Commelina communis*. They suggest that the phase dependent uptake of Cl^- and K^+ ions by the guard cells is a result of rhythmic changes in the guard cell membrane. The nature of these changes, however, remains uncertain.

Further evidence for the involvement of membranes in circadian clocks is the observations that many chemicals such as heavy water (D_2O), alcohol and valinomycin, which are known to affect membrane permeability, also affect the phase or period of circadian systems. For example, valinomycin, an ionophore, known to alter the permeability of the membrane to K^+, shifts the phase of the leaf movement rhythm in *Phaseolus multiflorus* (Bünning and Moser, 1972), and the phase response curve for valinomycin is similar to that for light. Phase shifts and period lengthening have also been found to occur in *Phaseolus* in response to D_2O (Bünning and Baltes, 1962). This chemical also lengthens the period of the rhythm in *Euglena* (Enright, 1971). In *Gonyaulax*, ethanol and valinomycin shift the phase of the luminescence rhythm (Sweeney, 1974).

If the central oscillating mechanism does involve changing ion gradients across membranes then an exogenous supply of ions might be expected to disrupt such gradients and thereby shift the phase or alter the period of a particular rhythm. Results of such investigations, at least in plants, have, on the whole, been negative. The phase and period of the rhythm of petal movement in *Kalanchoe blossfeldiana*, for example, was unaffected by brief or prolonged exposure to a solution containing K^+ ions (Schrempf, 1975); a similar finding was reported for

Gonyaulax. (Sweeney, 1974). Lithium ions have, however, been reported to lengthen the period of a number of rhythms, including those of K^+ uptake in *Lemna gibba* G3, (Kondo, 1984) and petal movement in *Kalanchoë blossfeldiana* (Schrempf, 1975). The significance of such results with respect to the mechanism underlying the generation of these rhythms must, remain uncertain, since Li^+ ions, in addition to influencing membrane permeability (Bose and Lowenstein, 1971), have also been reported to affect nucleic acid synthesis (Volm, Wayss and Schwartz, 1970).

Recent investigations have brought to light the possible involvement of membranes in the clocks of *Gonyaulax* and *Neurospora*, two organisms for which explanations for the generation of circadian rhythms had been sought in terms of protein or nucleic acid synthesis.

Johnson and Hastings (1986) have recently established that luminescence in *Gonyaulax* originates from discrete subcellular loci that are intrinsically fluorescent. These particles appear to be analagous to luminescent particles known as "scintillons" previously isolated *in vitro* (DeSa, Hastings and Vatter, 1963). The nature of these particles is unknown but it has been suggested that they may be membranous vesicles enclosing an acid milieu which provides the proton source known to be necessary to trigger luminescence. This idea would be consistent with a previous suggestion of Fogel and Hastings (1972) that "scintillon" flashing involved local acidification which stimulated the release of luciferin from its binding protein and activated luciferase.

Recent studies have also suggested that membranes may form an integral part of the clock in *Neurospora crassa*. Rhythms in the K^+ and Na^+ content of the growing front in this organism, have been reported (Sato, Kondo and Miyoshi, 1985), the K^+ rhythm corresponding in phase and period to the rhythm in conidiation. Moreover, additions of fatty acids to the medium altered the length of the period. It was not possible, however, to establish whether ionic changes triggered the rhythm in conidiation or whether the ionic changes were triggered

by an earlier stage of conidiation.

A rhythm of conidiation in a wild strain of *Neurospora crassa* can be induced by sodium lauryl sulphate (SLS) (Cramer-Herold, Lysek and Varchmin-Fuchs, 1986). These authors suggest that the plasmalemma plays a central role in the control of the rhythm in this organism, which they believe to be due to an increased proton influx into the hyphal cytoplasm. An immediate reaction to this acidification would be enhanced activity of the ATP synthetase. The effect of SLS is attributed to its incorporation into the lipid membrane, thereby weakening the structure and leading to an enhanced proton flux. Thus, it is possible that the basic oscillator in *Neurospora* consists of two parts; a biochemical component located in the plasmalemma which generates the oscillation and a regulating centre which dictates the circadian character of the oscillation.

The rhythm of CO_2 output in leaves of *Bryophyllum fedtschenkoi* has been extensively studied by Wilkins (1959, 1960a and b, 1962a and b, 1965, 1973, 1983). *Bryophyllum*, in common with many other succulent plants, exhibits crassulacean acid metabolism (CAM). The most recent review of CAM has been carried out by Ting (1985). During the hours of darkness CO_2 is fixed into oxaloacetate (OAA) under the action of phosphoenolpyruvate carboxylase (PEPCase). Malate dehydrogenase (MDH) then catalyses the reduction of OAA to malate which is transferred to, and stored in, the vacuole until daybreak. In the subsequent light period, malate moves out of the vacuole into the cytoplasm where it is decarboxylated to form phophoenolpyruvate (PEP) and CO_2. The PEP is then converted to storage carbohydrate whilst CO_2 is available for refixation by the operation of the Calvin cycle.

In recent years attention has focused on the mechanism of malate transport across the tonoplast, and the intracellular location of the enzymes involved in CAM.

Malate transport into and out of the vacuole has been extensively studied by Luttge's laboratory. It now appears that at least in *Kalanchoë daigremontiana*,

malate transport into the vacuole is an energy requiring process (Smith, Uribe, Ball, Heuer and Luttge, 1984; Smith, Uribe, Ball and Luttge, 1984). A proton pump driven by a tonoplast ATPase is proposed to be involved in the active transport of H^+ into the vacuole with $malate^{2-}$ following passively. Physiological and thermodynamic considerations have indicated that the ATPase activity associated with isolated vacuoles of *Kalanchoë* is sufficient to account for the observed rates of malic acid accumulation if the proposed proton pump operates with a stoichiometry of 1 ATP hydrolysed for every 2 H^+ transported. Malate efflux is thought to be a passive process; Luttge and Smith (1984) reported that efflux of malate occurred predominantly in the form of H_2malate.

One of the major difficulties in elucidating the steps in the CAM pathway has been the problem of the intracellular location of the enzymes involved. Whilst most of the evidence indicates that PEPCase is a cytosolic enzyme (e.g. Kluge and Ting, 1978; Spalding, Schmitt, Ku and Edwards, 1979; Winter, Foster, Edwards and Holtum, 1982), there have been a number of reports of the occurrence of this enzyme in the chloroplasts, (e.g. Schnarrenberger, GroB, Burkhard and Herbert, 1980; Perrot-Rechenman, Vidal, Brulfert, Burlet and Gadal, 1980) although none of the investigations eliminated the possibility that PEPCase had adhered to the outer chloroplast envelope during the extraction and fractionation procedure.

On the assumption that PEPCase is a cytosolic enzyme, it is likely that the reduction of OAA to malate is catalysed by an NAD-dependent malate dehydrogenase rather than the NADPH-dependent form. The latter enzyme is thought to be localised in the chloroplast and to be activated by light (Kluge and Ting, 1978; Gupta and Anderson, 1978) and thus probably not functional in the dark. NAD-dependent malic dehydrogenase is thought to be in the cytosol (see Kluge and Ting, 1978) although it may also be present in the mitochondria. (e.g. Schnarrenberger *et al.*, 1980; Mukerji and Ting, 1969,).

CAM plants may be divided into two groups on the basis of their decarboxylating enzymes (Dittrich, 1976; Edwards, Foster and Winter, 1982): Malic enzyme plants, to

which *Bryophyllum fedtschenkoi* belongs, and Phosphoenolpyruvate carboxykinase (PEPCK) plants. Both groups have sufficient activity of NADP and NAD malic enzyme, but malic enzyme plants are deficient in PEPCK. PEPCK has been reported in the cytosol and mitochondria (Edwards *et al.*, 1982; Kluge and Ting, 1975; Schnarrenberger *et al.*, 1980). The intracellular location of malic enzyme is difficult to establish since several isoenzymes are known to exist. NAD malic enzyme has been detected in the mitochondria (Wedding, 1982) and NADP-dependent malic enzyme in the cytosol and chloroplasts (Schnarrenberger *et al.*, 1980; Spalding *et al.*, 1979).

So, despite numerous investigations, there still remains a great deal of uncertainty about the location of enzymes in CAM and ultimately in the location of steps in the pathway. Further understanding of CAM will undoubtedly facilitate the understanding of the mechanism underlying the generation of the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* since there is little doubt that it arises from a rhythm in the operation of the CAM pathway. This rhythm appears to be due to the periodic activity of PEPCase (Warren and Wilkins, 1961). The end product of CO_2 fixation, malate, is a very effective feedback inhibitor of PEPCase activity.

Wilkins (1983) has accounted for the generation of this rhythm in continuous darkness and an air stream initially free of CO_2 in terms of the accumulation of malate in the cytoplasm followed by its effective removal to the vacuole. He suggests that during the first few hours of darkness, accumulation of malate in the cytoplasm inhibits PEPCase; this event is then thought to trigger the mechanism whereby malate is transported to the vacuole. When this happens the activity of PEPCase in the cytoplasm is restored and the sequence of events can begin again leading to the cyclic emission of CO_2 from the leaves.

This hypothesis has been extended to account for the characteristics of phase shifts induced by high temperature and light pulses in a similar manner to that

proposed by Njus *et al*. (1974) for the phase setting effects of light on the rhythm of leaf movement in *Phaseolus multiflorus*. Wilkins (1983) has proposed that on exposure to such treatments "gates" in the tonoplast open allowing the leakage of malate from the vacuole into the cytoplasm where it inhibits PEPCase. Shortly after the end of the treatment the "gates" are thought to close and the rhythm begins by malate being pumped into the vacuole. The magnitude of the phase shift depends upon how much malate leaks into the cytoplasm, which in turn depends upon the relative concentration of malate in the cytoplasm and the vacuole, and on the duration of the treatment. Those parts of the cycle over which high temperature or light treatments are effective in bringing about a phase shift are thought to represent stages where there is a significant malate concentration gradient existing between the vacuole and the cytoplasm. By allowing the leakage of malate, light or high temperature treatments of adequate duration will abolish such a gradient, whilst for shorter treatments the gradient will merely be reduced. As was mentioned earlier, in order to achieve a maximum phase shift the treatment must be of at least 3 h duration. It is deduced, therefore, that 3 h is the time required for sufficient malate to have leaked into the cytoplasm to abolish the malate concentration gradient across the tonoplast.

The peaks in the cycle of CO_2 output are thought to represent a stage just prior to the onset of the pumping of malate into the vacuole from the cytoplasm. At this stage there must be a high concentration of malate in the cytoplasm relative to the vacuole because PEPCase is inhibited. There will either be a small or no gradient across the tonoplast and as a result, a light or high temperature treatment is without effect because no net movement of malate from the vacuole to the cytoplasm will occur.

It has been suggested that the ability of inhibitors such as cycloheximide to abolish the rhythm of CO_2 exchange in *Bryophyllum* leaves, may be due to their effects on tonoplast permeability, and this again would result in the abolition of a

malate concentration gradient between the vacuole and the cytoplasm (Bollig and Wilkins, 1979).

Recently Buchanan-Bollig (1984) reported the occurrence of a rhythm of CO_2 uptake in leaves of *Kalanchoë daigremontiana* maintained in normal air and continuous illumination. A similar rhythm was subsequently found to persist under the same conditions in the leaves of *Bryophyllum fedtschenkoi* (Wilkins, 1984). In *Bryophyllum* the rhythm is very marked and persists for at least 10 days with an average period of 18.19 h ± 0.3 h at 15°C. This contrasts with the rhythm of CO_2 emission in leaves kept in a stream of CO_2 - free air and darkness which damps out after about 4 days and has a period of 23.4 ± 0.3 h. at 15°C.

The light rhythm is thought to arise from the same basic mechanism proposed for the dark rhythm. But, since it had been suggested that "gates" in the tonoplast open in response to a short light treatment, in continuous light the "gates" must be permanently open, so malate cannot be cleared from the cytoplasm by being pumped into the vacuole as it would simply leak back out again. It has therefore been proposed that removal of malate from the cytoplasm in continuous light occurs by metabolic breakdown, possibly involving malic enzyme which would produce pyruvate and CO_2. The latter product would be available for photosynthesis thus allowing the breakdown of malate to proceed to completion and the subsequent reappearance of PEPCase activity (Wilkins, 1984).

No explanation for the length of the period is provided by this hypothesis, but the difference in the length of the periods between the dark and the light rhythms may be due to the metabolic breakdown of malate in the light proceeding at a higher rate than the trans-tonoplast pumping mechanism thought to operate in the dark. If this were the case then the rate of malate breakdown in the light might be expected to increase with increasing light intensity, thus opening the possibility that the period of this rhythm is under the control of light intensity. This view would be entirely consistent with a recent finding by Buchanan-Bollig (1984) for

the rhythm of CO_2 exchange exhibited by leaves of *Kalanchoe daigremontiana* in light and normal air. The period of the rhythm in this plant was found to be a function of the light intensity, being 21.4 h at 60 W. m^{-2} and 24 h at 10 W. m^{-2}. In darkness and bright light (100 W. m^{-2}) no rhythm could be detected. Labelling studies with $^{14}CO_2$ indicated that in continuous light this rhythm was a consequence of PEPCase mediated CO_2 fixation and malate synthesis (Buchanan-Bollig, Fischer and Kluge, 1984), not as suggested by Queiroz (1979), due to rhythmic activity of both the carboxylating and the decarboxylating enzymes. Moreover, at high irradiances, which are known to reduce the levels of malic acid in the vacuoles to a permanently low level, the transfer of label into malate was found to be high, indicating that PEPCase mediated CO_2 fixation is not disrupted by high light intensity, but the storage of malic acid in the vacuoles is disrupted, a finding in accordance with Wilkins' hypothesis.

Whilst periodic activity of PEPCase brought about by the accumulation and removal of malate is thought to be the principle mechanism giving rise to the rhythm in CO_2 assimilation in *Bryophyllum* there are undoubtedly other contributing mechanisms.

The large changes of the activity of PEPCase *in vivo* cannot be accounted for in terms of diurnal changes in the capacity of this enzyme as they are invairiably very small (Jones, Buchanan, Wilkins, Fewson and Malcolm, 1981). However, it has recently been reported that PEPCase from leaves of *Bryophyllum fedtschenkoi* can exists in two forms (Nimmo, G., Nimmo, H., Fewson and Wilkins, 1984; Nimmo, G., Nimmo, H., Hamilton, Fewson, Wilkins, 1986); a night form, which is phosphorylated and has a low sensitivity to inhibition by malate, and a day form, which is dephosphorylated and is ten times more sensitive to inhibition by malate. The conversion of the day to the night form is accompanied by phosphorylation of one or more serine residues.

PEPCase from a number of other plants has also been found to exist in different forms (e.g. Winter, 1980a, 1982; Buchanan-Bollig and Smith, 1984) and phosphorylation of the enzyme has recently been reported in three species of *Kalanchoë* (Brulfert, Vidal, Marechal, Gadal, Queiroz, Kluge and Kruger, 1986).

More recently, Nimmo *et al.* (1987) have investigated the phosphorylation state of PEPCase from *Bryophyllum* leaves showing circadian rhythms under constant conditions. In CO_2 free air and darkness a persistent circadian rhythm of interconversion between the two forms of the enzyme was observed and a good correlation was found between the occurrence of the less malate-sensitive form and the times of maximum CO_2 fixation. In continuous light and normal air however, interconversion between the two forms of the enzyme occurred only when the onset of continuous light occurred at the end of the normal 8-h photoperiod. Although a persistent rhythm of CO_2 exchange was observed in plants placed in continuous illumination at the beginning of the 8-h photoperiod, no rhythm in the interconversion of the two forms of the enzyme was found. Thus it would appear that the variation in the activity of PEPCase brought about by covalent modification may contribute to the occurrence of the rhythm in CO_2 free air and darkness but its role in the generation of the rhythm in normal air and light remains uncertain.

Wilkins (1983, 1984) has advanced hypotheses to account for the generation of the rhythms in *Bryophyllum* leaves kept either in darkness and CO_2-free air or in light and normal air, for the induction of phase shifts in these rhythms by various environmental stimuli, and for the absence of a rhythm in leaves kept in darkness and normal air. These hypotheses are based on the change in the malate levels in the cells but they have never been subjected to critical examination.

The prolonged persistence of the rhythm of CO_2 exchange exhibited by leaves kept in light and normal air, together with its characteristically high amplitude, makes this rhythm an ideal one on which to test the validity of many aspects of

these hypotheses, and to relate the control of the rhythm to the malate status of the leaves.

1.6. AIMS OF THIS INVESTIGATION

The study reported in this thesis therefore, has a number of objectives. The first is to establish the detailed characteristics of the rhythm in leaves kept in continuous light and a normal air stream. In particular it is necessary to establish the temperature range over which the rhythm occurs and the extent to which the period is temperature-compensated. At any temperatures at which the rhythm is inhibited the nature of the inhibition will be investigated by restoring the temperature to within the functional range in order to establish whether or not a rhythm appears and if so whether it begins from a fixed phase point. This information will enable deductions to be made about the likely malate status of the leaves during the time at which the rhythm is inhibited. The validity of these deductions can then be established by actually determining the malate status of the leaves. If these investigations provide evidence which supports the concept that malate levels are involved in both the generation and phase control of the rhythm, they could be broadened to determine whether or not a variety of other environmental parameters which might be expected to affect the malate status of the leaves act in a predictable way to inhibit the rhythm or shift the phase.

2. MATERIAL AND METHODS

2.1. PLANT MATERIAL

The experimental material used throughout this investigation was the detached leaves of the succulent plant *Bryophyllum*(*Kalanchoë*) *fedtschenkoi* (R. Hamet et Perrier). The stock of plants originated from a single plant obtained from the Royal Botanic gardens, Kew, in 1955 and had been reproduced vegetatively since that time to produce a clone of material which was identical to that used by Wilkins (1959, 1960a, 1960b, 1962a, 1962b, 1965, 1967, 1973, 1983, 1984). A continuous supply of experimental material was provided by taking cuttings from stock plants at 8-week intervals. The cuttings consisted of stem apices, 5 - 6 cms in length, having about five pairs of leaves. They were grown in 10 mm diameter plant pots in a compost mixture of coarse sand: SAI potting compost: topsoil (1: 1: 1), to which 0.05 of a part of each of ENMAG and nitrochalk were added. Three or four cuttings were planted in each pot. The plants were maintained at a minimum temperature of 18^{0}C in a glasshouse where a 16-h photoperiod was maintained throughout the year by use of mercury vapour lamps during the winter months. The cuttings were grown under these conditions for about 4 months.

At least 7 days, and usually 2 - 3 weeks, before being required for experimental purposes, plants were transferred to a controlled-environment room where they were maintained under short-day conditions. A bank of Phillips fluorescent lamps (daylight : warm white 1:1) and twelve, 100 W, tungsten lamps, giving a quantum fluence rate of 110 μ mol m^{-2} s^{-1} at half plant height, provided an 8-h photoperiod. The temperature of the room was maintained at 25 ± 0.5^{0}C during the photoperiod

and 15 ± 0.5°C during the dark period. Two such rooms were available; the lighting and temperature conditions in each were similar but in one the photoperiod extended from 0800 h to 1600 h while in the other, the time of the photoperiod was usually 1600 h to midnight. For a few experiments, however, the timing of the 8-h photoperiod in the second controlled-environment room was changed to provide material having a rhythm in which particular phase points occurred at convenient times of the day. Plants were watered every second day.

Experiments were carried out on large (6 x 4 cm) succulent leaves showing slight purple pigmentation. The production of suitable plant material was promoted by removing stem apices showing signs of flowering. Such apices are readily distinguishable because their orientation changes from being upright to being pendulous.

It has been reported that young leaves of *Bryophyllum fedtschenkoi* may not show typical crassulacean acid metabolism (Jones, 1975), so the top five pairs of leaves on a shoot were not used. Only fully expanded leaves at least 6 pairs down from the shoot apex were used. Those showing signs of senescence were rejected, as were any that had been damaged.

Under the conditions used to grow the plants, no plantlets developed at the leaf margin.

2.2. METHODS

2.2.1. THE MEASUREMENT OF CO_2 EXCHANGE

The rate of CO_2 uptake or emission by the leaves was recorded automatically using an ADC infra-red gas analyser (IRGA) (ADC 225 mark 3, Analytical Development Company Ltd., Hoddeson, Herts, U.K.) incorporated into the apparatus

shown diagramatically in Fig. 2.2. A photograph of the system is presented in Fig. 2.3.

A diagram of the principal components of an infra-red gas analyser (IRGA) is provided in Fig. 2.1. The principle of operation of the IRGA is relatively simple. Infra-red radiation from the infra-red source (IRS) is directed through the sample (S) and reference (R) tubes of the IRGA and measured by the detector (DC). The detector is a closed container consisting of two compartments filled with CO_2 and separated from each other by means of a flexible metal diaphragm. Infra-red radiation passing through the reference cell enters one compartment of the chamber and that passing through the sample cell enters the other. As the CO_2 in each chamber of the detector absorbs the infra-red radiation it is heated, and the gas therefore expands. A difference in the amount of infra-red radiation reaching the two sides of the detector causes the gas in the two compartments to be at different temperatures and the resulting pressure difference displaces the flexible metal diaphragm. This diaphragm acts as one plate of an electrical condenser so that its movement towards or away from the other rigid, but perforated plate, alters its electrical capacitance, and this change is detected in the electronic circuit of the analyser, amplified, (A) and recorded on the meter (M). The displacement of the diaphragm is proportional to the difference in the CO_2 concentration in the sample and reference cells of the analyser.

Reference to Fig. 2.2 shows that air from a compressed air cylinder (British Oxygen Co Ltd , Polmadie Road, Glasgow, G5) was first passed over a column of water (E) which acted as a safety valve and maintained a constant pressure in the system. The air flow was then divided into four separate streams each of which passed to a capillary flowmeter (F1 - F4). The flowmeters, which were mounted on a measuring scale, were constructed from glass manometer tubes filled with Brodie's solution (Philip Harris Ltd., Lyne Lane, Shenstone, Staffordshire) with a U-shaped capillary

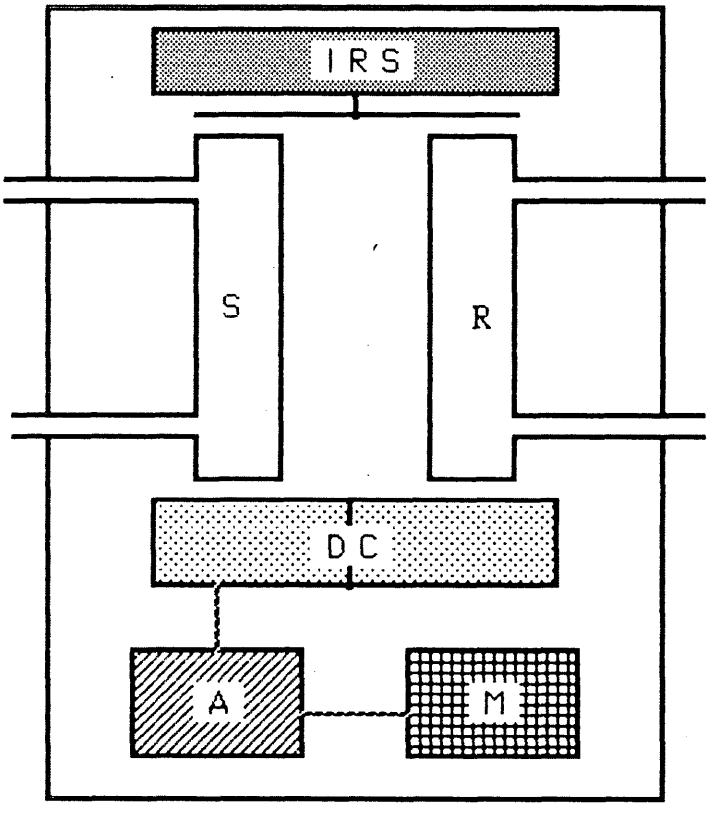
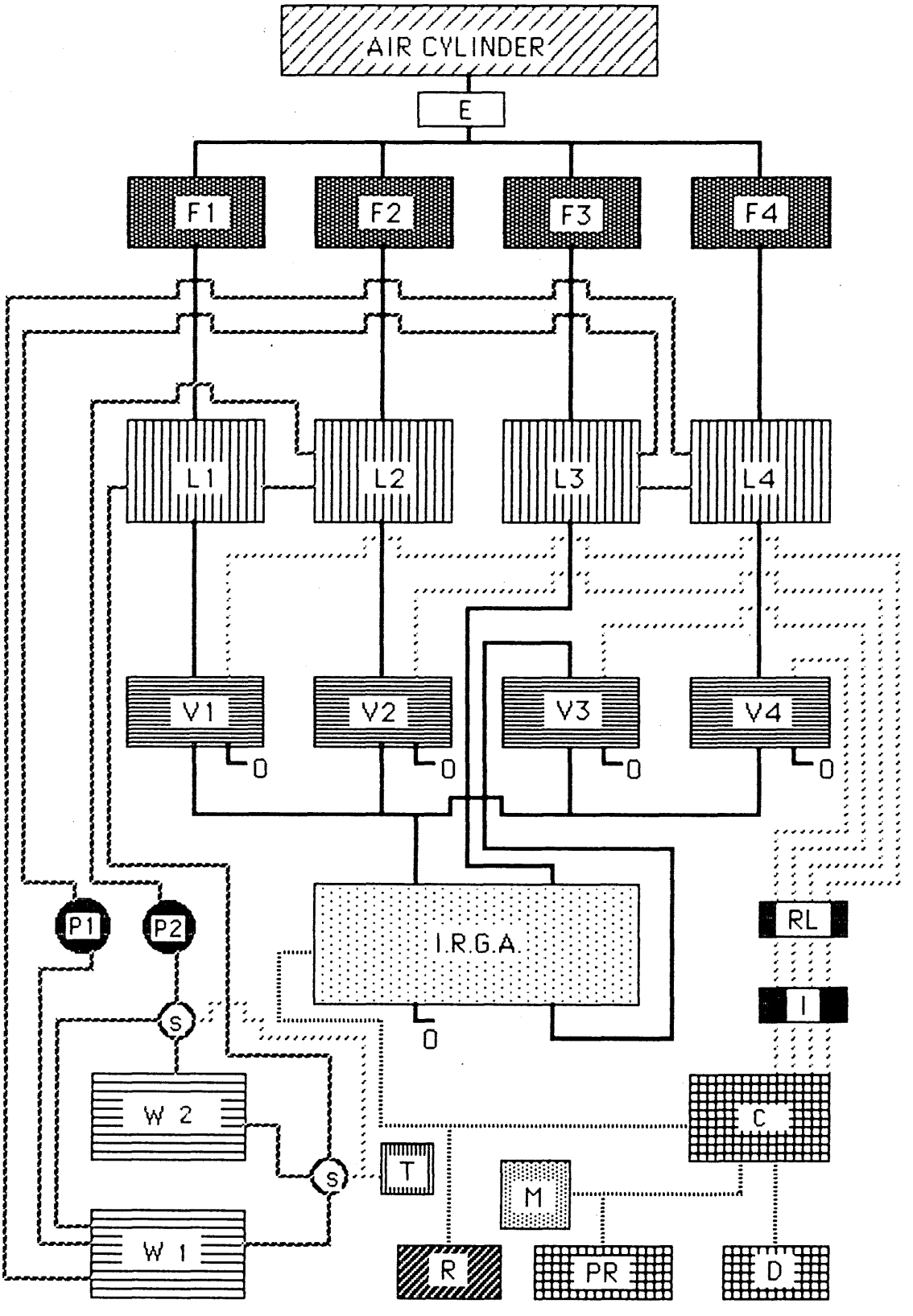


FIGURE 2.1. Diagram of Infra-Red Gas Analyser

IRS	Infra-Red source
S	Sample cell
R	Reference cell
DC	Detecting condenser
A	Amplifier
M	Meter

Figure 2.2. Diagram to show layout of apparatus used to record the rhythm of carbon dioxide exchange in leaves of *Bryophyllum fedtschenkoi*

E	Safety valve
F1-F4	Flowmeters
L1-L4	Leaf chambers
V1-V4	Microsolenoid valves
O	Waste
IRGA	Infra-red gas analyser
P1 & P2	Pumps
W1&W2	Water bath
T	Timer
S1&S2	Solenoid valves
R	Chart recorder
M	Monitor
PR	Printer
C	Computer
D	Disc drive
I	Interface
RL	Relay valves
———	Gas flow
- - - - - - - - -	Water flow
.....	Command signals
.....	Data collection


AIR CYLINDER
E
F1
F2
F3
F4
L1
L2
L3
L4
V1
V2
V3
V4
O
O
O
O
P1
P2
I.R.G.A.
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RL
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T
C
M
W 1
R
PR
D

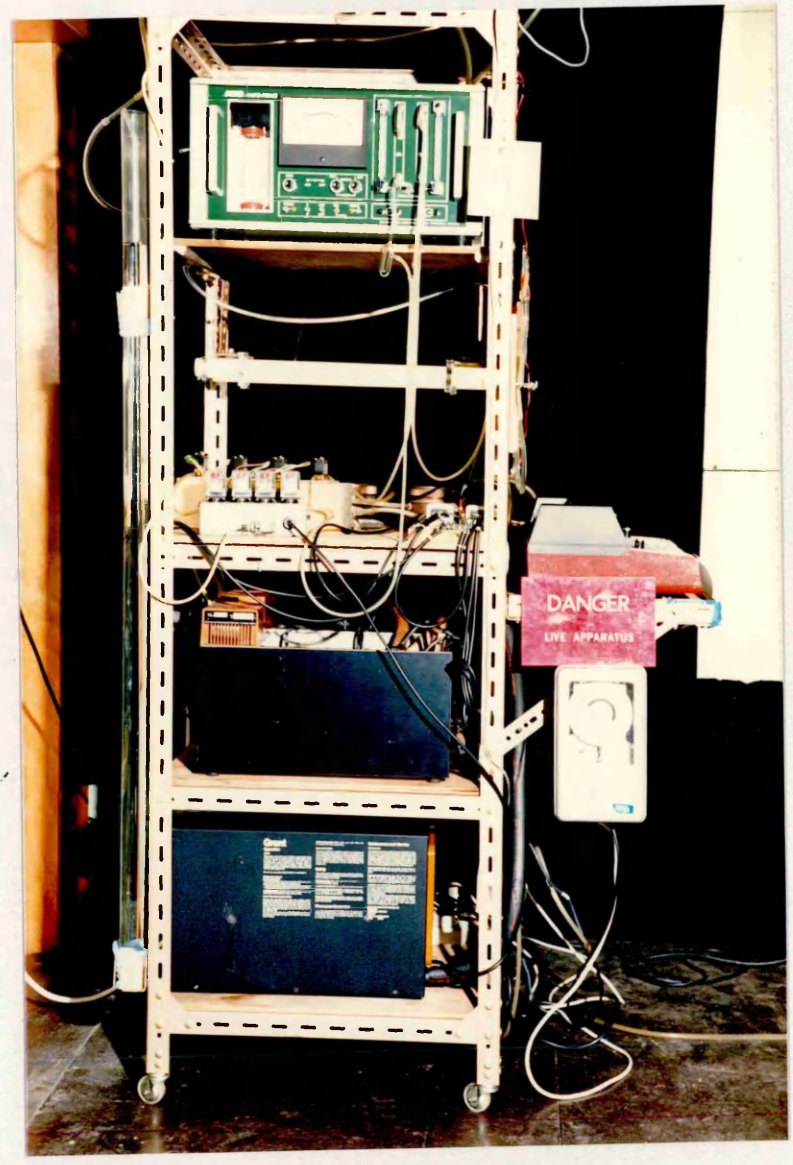


FIGURE 2.3. Photograph of the apparatus used to record the rhythm of CO_2 exchange.

tube attached to the open ends by means of short lengths of rubber tubing. The resistance to flow induced by the capillary as the air-stream passed from the inlet to the outlet caused a displacement of the solution which was proportional to the gas flow rate. The rate of flow, which was maintained at either 1.5 or 3.0 l h^{-1} in all experiments, was controlled at two points in the system: at the cylinder head by means of fine, gas-flow, regulator valves, to give coarse control, and at the polythene tubing inlet to the manometer by means of 3 cm screw clips, to give fine control.

The air stream which emerged from flowmeters 1, 2 and 4 passed through a leaf chamber (L1, L2 and L4) and thence to a Bürkert, three-way, micro-solenoid valve (V1, V2 and V4) (Bürkert Contromatic Ltd, Stroud Glos., U.K.). Air from flowmeter 3 was used as reference gas and after passing through leaf chamber L3, entered the reference cell of the IRGA before being directed into a three-way solenoid valve, V3. The solenoid valves were activated in sequence so that they each directed their air stream into a manifold which led to the sample cell of the IRGA for 15 min, and to waste (0) for the remaining 45 minutes, in each hour.

Activation of the valves was achieved by a BBC microcomputer (C) (model B. Commscot, Glasgow, U.K.) which was programmed to activate the valves in a strict sequence. The activating signal from the computer was amplified in an interface (I) from which a 12 V signal was transmitted through one of the 4 lines shown in Fig. 2.2 to one of the four relays (RL). Activation of a relay sent a mains voltage (240 V) to the appropriate solenoid valve (V1- V4). On being so activated, the solenoid valve directed its air stream into the sample cell of the IRGA. When it was deactivated the gas escaped to waste (0).

The output signals from the IRGA represented the difference in CO_2 concentration between the air in the reference and sample cells of the IRGA and were plotted automatically against time on a Linseis, flat-bed, chart recorder (R) (Series L600, Belmont Instruments, 2 Clairmont Gardens, Glasgow, G3 7LW). Because

the gas flowing through the sample cell of the gas analyser changed its origin every 15 mins, the data recorded on the chart recorder appeared as a series of steps. The sequence was arranged so that for the first 15 min in each hour gas from leaf chamber 1 was analysed, for the second 15 min that from chamber 2, for the third 15 min that from chamber 3 and for the final 15 min that from chamber 4. Chamber 3 was always blank (no leaves) and provided the base line from which the rate of CO_2 uptake or emission by leaves in the other chambers could be calculated. Readings taken from the chart were made at the end of the 15 min period, after a stable reading had been attained by the gas stream fully purging the sample cell of the gas analyser. The chart data were collected only as a back-up because the data were principally collected and processed by the computer. The computer was programmed to collect and average the IRGA output signals over 1 min, just before the end of each 15 min sampling period. It then processed the data for each leaf sample in the following way: first, the blank reading from chamber 3 was subtracted from the sample reading and the resultant value was multiplied by a "correction factor", calculated by dividing the calibration value of the IRGA by the fresh weight of the leaves. The resultant values which represented the rate of CO_2 uptake or emission by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$ were displayed on the monitor (M) every fifteen minutes and stored by the computer on floppy discs (D). In addition they were recorded automatically against time by the printer (PR) (Radioshack TRS-80 Tandy Corporation, Glasgow, U.K.).

The structure of a leaf chamber is shown in Fig. 2.4 and in the photograph in Fig. 2.5. These chambers were specially made, gas-tight, brass containers (internal diameter 6 cm) with a removable perspex and brass top. The air-stream was conducted into and out of the chambers via 3 mm, brass, conducting tubes (GI & GO). Each chamber was surrounded by a brass water jacket (W) through which water was continuously circulated from a Grant's, constant-temperature water bath

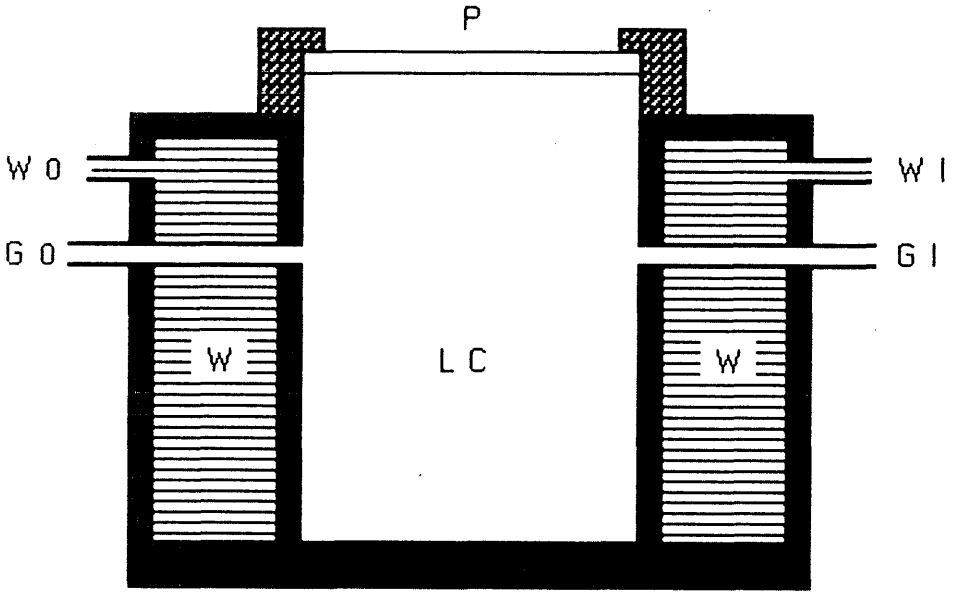


Figure 2.4. Diagram of a leaf chamber

L C	Leaf chamber
P	Lid
W	Water jacket
WI	Water in
WO	Water out
GI	Gas in
GO	Gas out

FIGURE 2.5. Photograph of leaf chamber containing leaves.

(Grant Instruments, Barrington, Cambridge, CB2 5Q2. England). Bath W1 in Fig. 2.2 was always maintained at 15 ± 0.1°C, but the temperature of bath W2 was varied according to the needs of the experiment. The temperature of the water baths was regulated by means of a thermostat, and a cooling coil from a Grant's refrigeration unit which was inserted into each bath.

The basic apparatus was changed from time to time according to the requirements of the experiments. In an extensive series of experiments the leaves were subjected to a number of different temperature regimes. To achieve these, water was pumped from the water baths and circulated to the leaf chambers by means of two pumps (Stuart Turner Ltd., Henley-On-Thames, England). Pump P1 (Fig. 2.2) pumped water from the 15°C water bath (W1) to leaf chambers 3 and 4 and back to bath W1. Leaves placed in chamber 4 were thus subjected only to a circulating water temperature of 15°C, and the rhythm exhibited by these leaves acted as the control rhythm in all experiments. A second pump, P2, circulated water to leaf chambers 1 and 2 and could draw its supply from either of the two water baths. The bath from which water was pumped, and to which it was returned, by this pump was controlled by means of two ASCO solenoid valves (S1 and S2 in Fig. 2.2) (ASCO., U.K. Ltd., 2 Pit Hey Place, West Pimbo, Skelmersdale, Lancs, WN8 9PG) connected in parallel to a time switch (T). When both these valves were activated simultaneously, water was pumped from bath W2 through valve S1 to leaf chambers L2 and L1 in series and then returned to the bath through a second valve S2. This second valve ensured that the water was returned to the bath whence it came. When the solenoid valves were deactivated, pump P2 circulated water to leaf chambers L1 and L2 in the same manner as described above except that instead of pumping water from bath W2, water was pumped from, and returned to, bath W1 (i.e. 15°C).

Activation of the solenoid valves S1 and S2 was controlled by means of a Venner, multi-set, electric time control (T) (model 42011-21. BDH., Burnfield Avenue,

Thornliebank, Glasgow). Thus, by setting the timer to activate the valves at the appropriate time and for the appropriate duration, leaves in chambers L1 and L2 could be subjected to pulse-type and step-type temperature changes.

In experiments which required a circulating water temperature of greater than 25°C, some water condensed in the polythene tubing conveying the gas from the leaf chamber to the solenoid valves. To overcome this problem, glass water traps were inserted into the gas system between each leaf chamber and its corresponding solenoid valve. Two further traps were incorporated into the gas system immediately before entry to the sample and reference cells of the IRGA; these traps served as safety devices to catch any manometric liquid which might be blown through the gas system should a sudden increase in the gas pressure occur.

The leaves were continuously illuminated from above by means of two 20 W Phillips warm white, fluorescent tubes placed about 20 cm above the leaf chambers, the distance being adjusted to give a quantum fluence rate of 11 - 12 μmol m^{-2} s^{-1} at the leaf level. Quantum fluence rates were measured with a quantum measuring system (type SKP 215, Skye Instruments Ltd., The old manse, Skedbost Bridge by Portree, Isle of Skye, Scotland, 1V51 9XE.

There were four sets of analytical apparatus, A, B, C and D, each controlled by the BBC microcomputer as shown in Fig. 2.6. Some aspects of the sets of apparatus were, however, different because of different experimental requirements. Two identical sets of the apparatus were arranged as in Fig. 2.2, both sets were used in experiments where a temperature change was required. One set, A, was used to study the effects of high temperatures on the rhythm and the other, B, to study the effects of low temperatures. The water flow system in both A and B was later modified however to enable leaf samples contained in chambers 1 and 2 to be subjected to temperature treatments of different durations. This modification is shown in Fig 2.7. Essentially two extra ASCO solenoid valves and one further timer were incorporated into each set of apparatus and the water supply to chambers 1

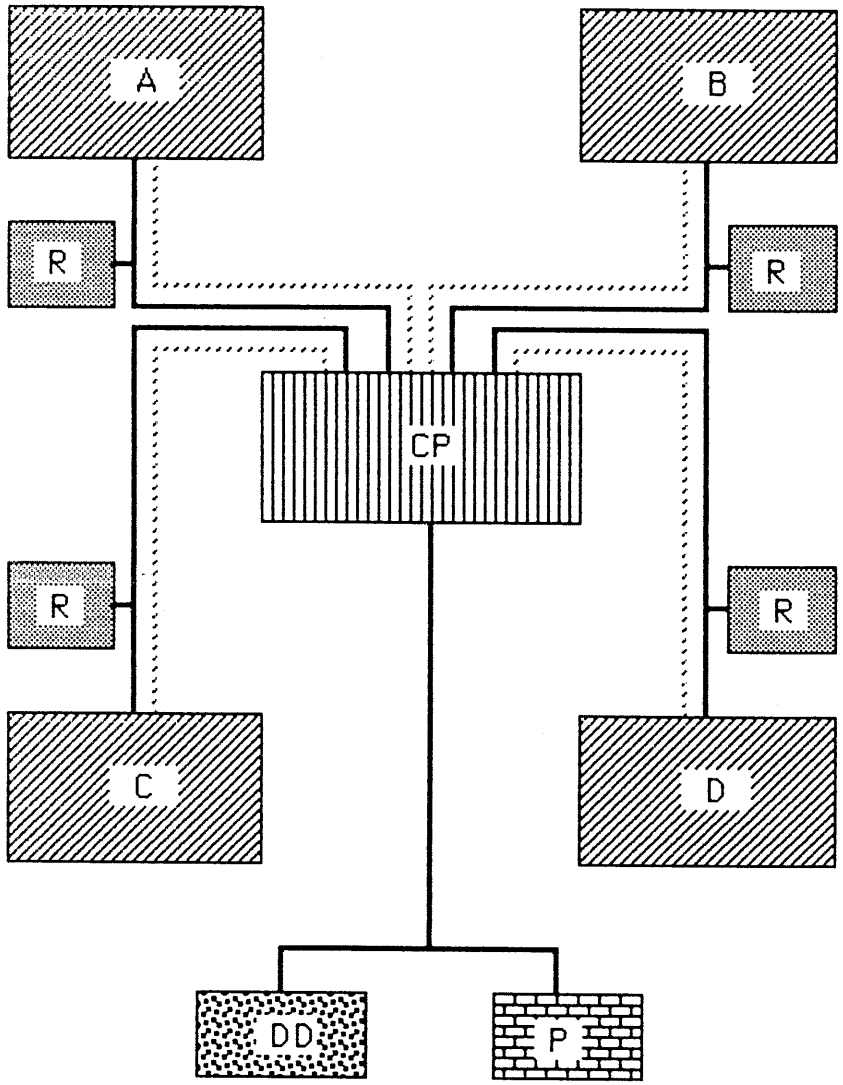


Figure 2.6. Diagram to show arrangement of apparatus.

A, B, C & D	Apparatus
R	Recorder
Cp	Computer
DD	Disc drive
P	Printer
———	Data collection
.....	Command signals

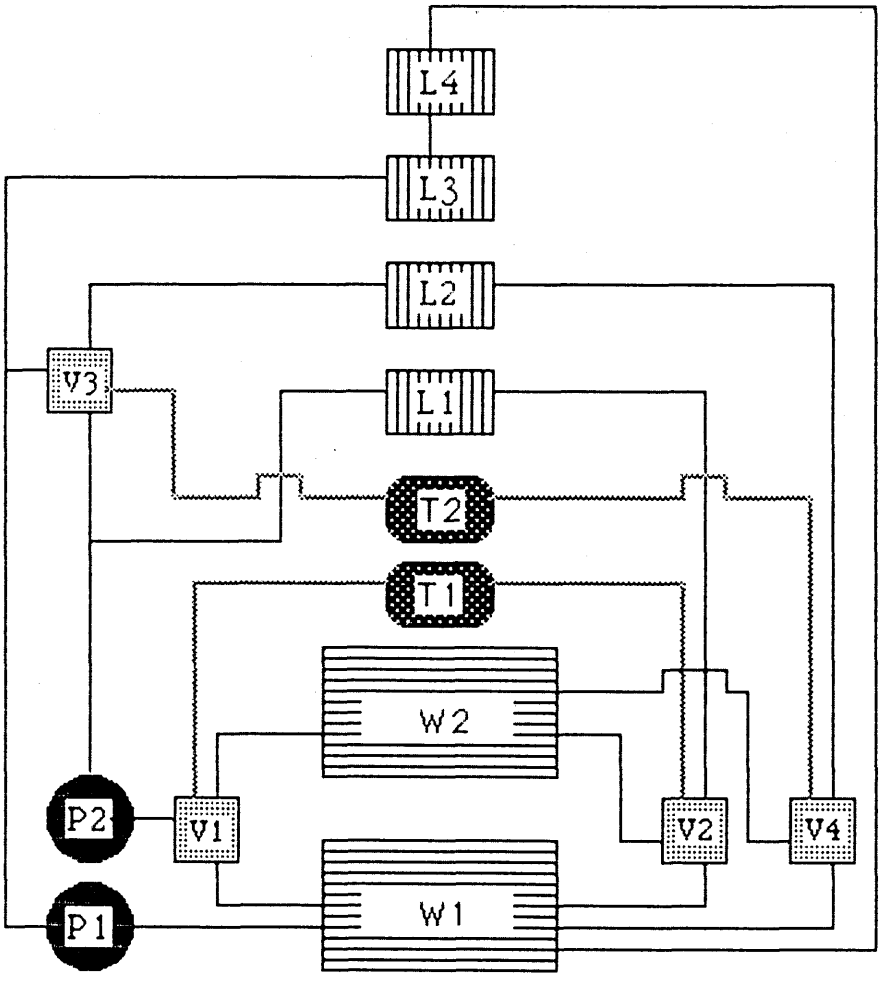


Figure 2.7. Diagram to show water flow during temperature treatments of different durations.

T1 & T2	Timers
L1 - L4	Leaf chambers
V1 - V4	Valves
P1 & P2	Pumps
W1 & W2	Water baths
———	Water Flow
~~	Electrical connections

and 2 separated. Timer T1 controlled valves V1 and V2 and timer T2 controlled valves V3 and V4. When all valves were inactive pump P2 circulated water from bath W1, which was always maintained at 15°C, to leaf chamber L1 and returned it to the same bath through valve V2. Pump P1 pumped water from bath W1 and circulated it to leaf chamber L2 via valve V3 and back to the same bath through valve V4. This pump also circulated water through leaf chambers L3 and L4 in series before returning it to bath W1. When timer T1 was set to activate valves V1 and V2, water from bath W2, which was maintained at 40°C in apparatus A and 2°C in apparatus B, was circulated to leaf chamber L1 and back to bath W2 through valve V2. Activation of valves V3 and V4 by timer T2 allowed water from bath W2 to be circulated through leaf chamber L2 and returned to bath W2. It was only possible to activate valves V3 and V4 when valves V1 and V2 were also activated. Activation of V3 and V4 alone would have resulted in water being pumped from bath W1 to leaf chamber L2 and then being returned to bath W2. Although this system did enable leaves in chambers L1 and L2 to be exposed to either high temperatures in apparatus A, or low temperatures in apparatus B, for different durations, it was essential that the treatments ended at the same time. This limitation was however fully acceptable for the purposes of the experimental programme.

The effects of a number of constant ambient temperatures on the rhythm were investigated using an earlier version of the apparatus shown in Fig 2.2 which incorporated only a single water bath and cooling coil. Water at a constant temperature was circulated through each of the four leaf chambers in series before being returned to the bath.

The two other sets of apparatus, C and D, while being closely similar to that shown in Fig 2.2, were specially modified to enable the effects of darkness and high concentrations of CO_2 on the phase and period of the rhythm to be studied.

2.2.1.1. Dark Apparatus (C)

The principal modification in this set of apparatus (C) was in the lighting system, a diagram of which is shown in Fig. 2.8. Each of the three leaf chambers, L1, L2 and L4 was enclosed within a separate light-tight box to exclude all extraneous light. In each compartment, a single 80 cm white fluorescent tube (Phillips warm white), was suspended 15 - 20 cm above the leaf chambers to give a quantum fluence rate of 11 - 12 $\mu mol\ m^{-2}\ s^{-1}$ at the level of the leaf surface. The fluorescent tubes in the first two compartments were separately controlled by Venner, multi-set, electric timers so that leaves in leaf chambers L1 and L2 could be exposed quite independently to dark periods of any duration at any time of day. Leaves in chamber L4 in the third compartment were maintained under continuous illumination and served as a control. All experiments in this set of apparatus were conducted at a circulating water temperature of 15 ± 0.1°C from a single water bath.

A further minor difference between this apparatus and the one shown in Fig. 2.2 was that a Grubb Parsons IRGA (Type 120. NEI Electronics Ltd, Whitely Road, Longbenton, Newcastle Upon Tyne, England, NE12 9SP) was used in place of the ADC IRGAs incorporated into the other 3 sets of equipment.

2.2.1.2. Apparatus for Investigating the Effects of High CO_2 Concentrations on the Rhythm

The modification to the gas flow system which enabled the effects of high concentrations of CO_2 on the rhythm to be studied is shown diagramatically in Fig. 2.9, and in the photograph in Fig. 2.10. Two three-way ASCO solenoid valves were inserted into the gas system at the entry and exit to each of the two sample leaf chambers. When these valves were deactivated, the normal air stream from the

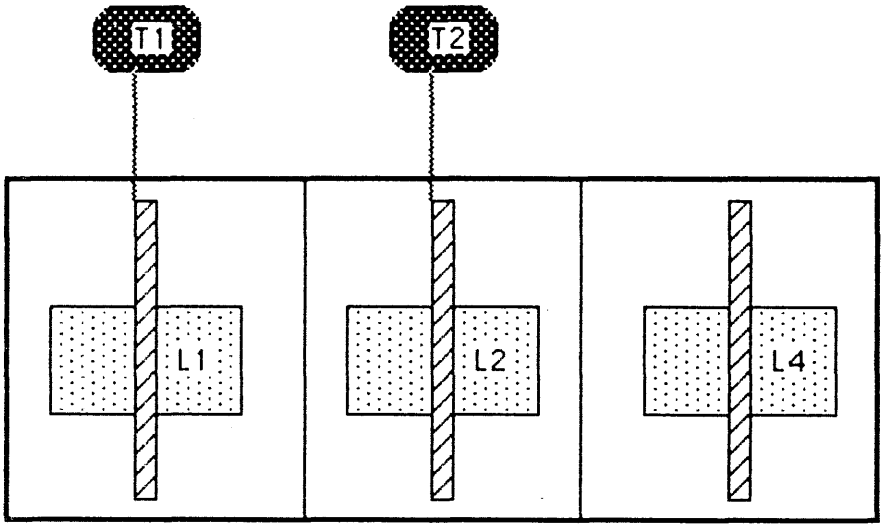


Figure 2.8. Diagram to show lighting system in apparatus C

T1 & T2	Timers
L1, L2 & L4	Leaf chambers
	Fluorescent tubes

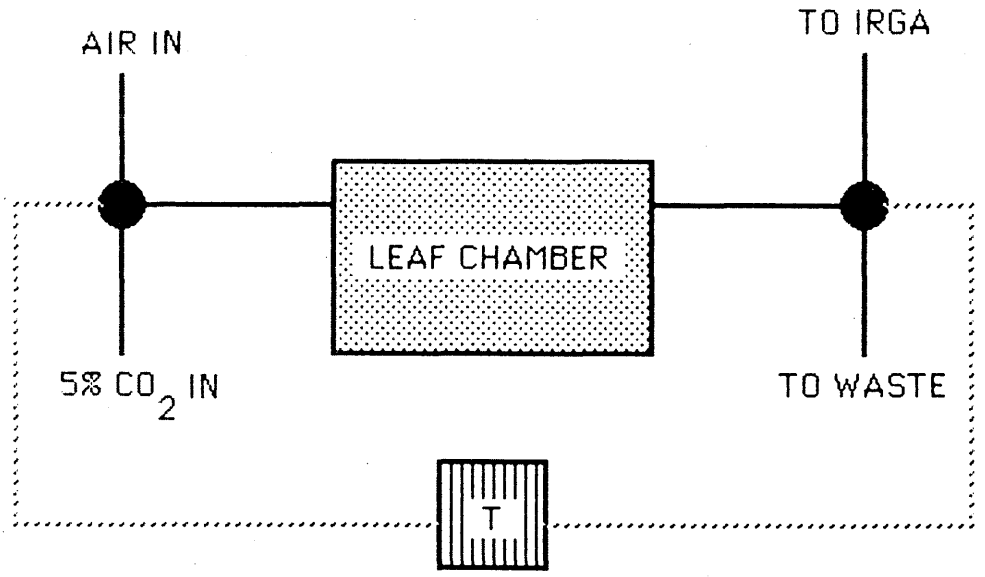


Figure 2.9. Diagram to show gas flow during carbon dioxide treatments.

●	Valve
T	Timer
———	Gas flow
.....	Electrical connections

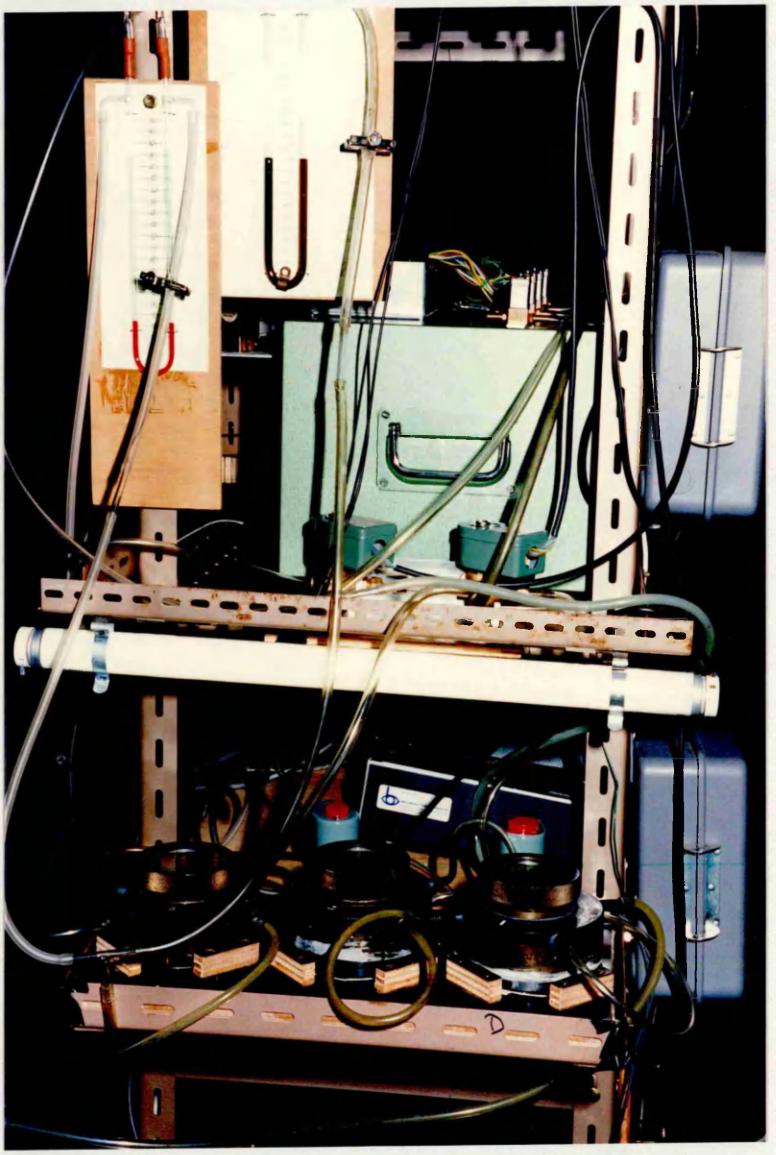


FIGURE 2.10. Photograph of apparatus used to investigate the effects of high concentrations of CO_2 on the rhythm of CO_2 exchange.

cylinder flowed via the solenoid valve to the leaf chamber and thence to the IRGA. To provide a 5% CO_2 treatment, both valves were activated simultaneously by the timer (T). Activation of the valves located at the entrance to each leaf chamber resulted in the normal air supply to the chambers from the air cylinder being cut off whilst the gas containing 5% CO_2 from a second cylinder (British Oxygen Company (Special Gases), Deerpark Road, London) was allowed to pass through the chambers. Activation of the valves located at the exit to each chamber ensured that during a 5% CO_2 treatment, the effluent from each chamber was directed to waste. This was necessary to prevent the high concentration of CO_2 entering the sample tube of the IRGA and damaging the highly sensitive detecting condenser in the analyser which normally operates at ppm levels. Each pair of solenoid valves associated with the two sample leaf chambers were connected to separate Venner, multi-set, time switches, providing flexibility to activate the valves independently so that leaves could be exposed to 5% CO_2 at different times of day and for various durations. The rate of gas flow during a 5% CO_2 treatment was maintained at 3.0 l h^{-1} by means of two capillary flowmeters. The normal air flowed through each of the chambers at a rate of 1.50 l h^{-1}. Leaves in chamber L4 were maintained in normal air throughout an experiment to provide the control sample.

In all experiments involving treatment of the leaves with 5% CO_2, the temperature of the circulating water was maintained at 15 ± 0.1°C, and continuous illumination was provided by a single Phillips 20 W, warm white, fluorescent tube placed above the leaf chambers to give a quantum fluence rate of 11 - 12 µmol m^{-2} s^{-1}.

All four sets of apparatus were kept in a dark room in which the temperature was controlled to within ± 3°C of a mean value which was set at 20°C.

2.2.1.3. Experimental Procedure

In setting up experiments the following procedure was adopted. Leaves were detached from the parent plants between 1530 h and 1600 h at the end of the photoperiod. They were weighed, and either one or two placed in each chamber with their cut ends in a 3 cm petri dish containing distilled water. The leaf chambers were securely closed and the "correction factors" for each leaf sample, obtained by dividing the calibration value of the IRGA by the fresh weight of the leaves, were entered into the computer.

2.2.2. MALATE DETERMINATIONS

2.2.2.1. Preparation of Leaf Extracts

Two methods were compared for the preparation of leaf extracts.

Method 1. Leaves were squeezed through a garlic press and the sap collected. Leaf debris was removed by centrifugation (MSE microcentaur) at 11,600 g for 2 mins. The whole procedure was carried out in a cold room at 4^0C and the leaf extracts were either assayed immediately or frozen until required.

Method 2. Leaves were cut into small pieces and homogenised for 2 mins in 5 volumes of 6% (v/v) ice cold perchloric acid with 4 drops of octan-1-ol in a 20 ml capacity MSE Atomix blender. The homogenate was centrifuged at 11,600 g for 1 min to spin down the leaf debris. A 2 ml sample of the supernatant was taken and neutralised to pH 5 - 7 with KOH. The precipitate of potassium perchlorate was removed by centrifugation at 11,600g for 2 min and the resultant supernatant containing the malate was used for the assays.

2.2.2.2. Malate Assay

Malate was determined spectrophotometrically using the method described by Möllering (1974). NADH formation, as measured by the increase in extinction at 340 nm is proportional to the amount of malate present. All measurements were made on a Pye Unicam SP8-50 spectrophotometer (Pye Unicam Ltd., York Street, Cambridge, CB1 2PX, England).

The standard assay mixture, in a final volume of 1 ml contained:

820 µl 100 mM Bis-tris propane buffer (pH 9.5).

100 µl 0.5 M Glutamate (pH 10).

75 µl Nicotinamide-adenine dinucleotide (NAD), Grade II 98% free-acid

2 µl Glutamate-oxaloacetate-transaminase (GOT). Pig heart suspension in ammonium sulphate, 25 mg ml^{-1}.

2 µl Malate dehydrogenase (MDH). Pig heart suspension in glycerol, 25 mg ml^{-1}

1 µl Leaf extract

The enzymes (GOT and MDH) and the NAD were obtained from Boehringer Corporation (Mannheim House, Bell Lane, Lewes, East Sussex BN7 1LZ) and the Bis-tris propane buffer and glutamate from Sigma Chemical Company (Fancy Road, Poole, Dorset, BH17 7NH).

The blank cell contained all the components of the system except the MDH. MDH was added to start the reaction which was complete within 2-3 min. Duplicate assays were carried out for each leaf extract.

Although not actually used in the determination of malate concentrations, a calibration curve was constructed by adding 1 µl of malate solutions (Sigma Chemical Company) of different concentrations in place of the leaf extract, to the assay mixture. This curve is shown in Fig. 2.11. and was used to check that the relationship between the increase in extinction at 340 nm and the quantity of malate present in the assay mixture was linear.

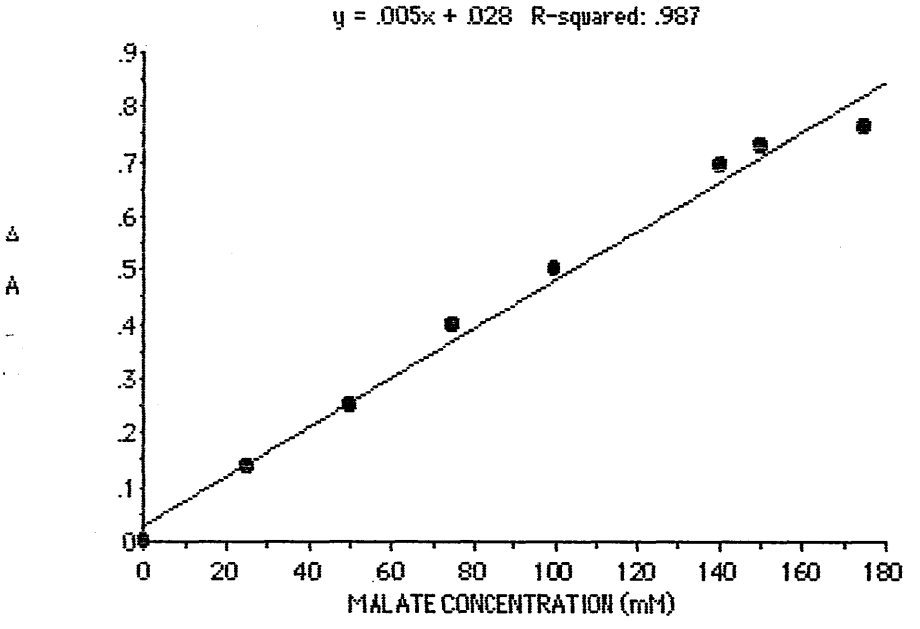


FIGURE 2.11. Malate calibration curve. Each point represents the mean of two readings. Ordinate: Absorbance change at 340 nm. Abscissa: Malate concentration of the stock solutions in mM.

2.2.2.3. Experimental Procedure

Leaves were excised from the parent plants in the second controlled-environment room at the end of the 8-h photoperiod. As previously mentioned, the timing of the photoperiod in this room was changed so that particular phase points in the rhythm occurred at convenient times of day. Plants were allowed to adapt to a change in the timing of the photoperiod for at least 5 days before being used in experiments. Leaves were placed in the leaf chambers under the lighting, temperature and gas composition conditions required by the experiment. Two leaves were placed in each chamber and were removed 1 min prior to extraction. Where malate curves are presented, each point on the curve represents the mean concentration of malate in the extracted cell sap of about 12 leaves obtained by assaying each individual leaf separately.

2.2.3. CALIBRATION OF THE APPARATUS

2.2.31. Infra-Red Gas Analyser

The manufacturer's calibration value was checked by means of a standard cylinder of calibration gas containing 25 ppm CO_2 in Nitrogen (Air-products Ltd., Langmuir Road, Bargeddie, Glasgow.). CO_2-free air, obtained by passing normal air through a series of 3 flasks containing 10% KOH was directed through the sample and reference tubes of the IRGA until a reading of zero was recorded on the scale. The calibration gas was then passed through the sample cell of the IRGA and the reading on the scale recorded. This procedure confirmed the values provided by the manufacturer. The calibration value could also be checked regularly by means of the automatic calibration check built into the IRGA. At a gas flow rate of 3.0 l h^{-1} it was calculated that one division on the IRGA meter was equivalent to a rate of

CO_2 output or uptake by the leaves of 5.4 µg CO_2 h^{-1} g (fresh weight)$^{-1}$ for the ADC IRGAs and, 11.98 µg CO_2 h^{-1} g (fresh weight)$^{-1}$ for the Grubb Parsons' IRGA. At a flow rate of 1.50 l h^{-1} these calibration values were halved. The method of calculating these values is set out in detail in the appendix. All IRGAs were used on the differential mode.

2.2.3.2. Capillary Flowmeters

The flowmeters were calibrated by recording the time taken for air flowing through them at different pressures to displace water from a 250 ml volumetric flask. Fig. 2.12 A, B, C and D show the values obtained for the flowmeters of apparatus A. The results obtained by this procedure were confirmed and routinely checked by a calibrated flowmeter (British Oxygen Company, Polmadie Road, Glasgow, G5).

2.2.4. TREATMENT OF DATA

The data collected and stored on discs by the BBC computer controlling the apparatus were processed by a second BBC microcomputer (model B). The second computer was programmed to drive a ColourPro Graphics Plotter, (Model 7440A, Hewlett Packard Ltd., Trafalgar house, Navigation Road, Altrincham, Cheshire, WA14 1NU) which plotted out at hourly intervals the rate of CO_2 uptake or output against time for each leaf sample and joined each point to produce a continuous curve.

The phase of the rhythm was assessed by the time of occurrence of the peaks in CO_2 exchange. The time at which the peaks occurred was estimated by taking the midpoint of the horizontal width of a peak at half-peak height. The period of the rhythm was determined by measuring the mean time between the occurrence of successive peaks. Phase shifts were assessed by comparing the times of occurrence

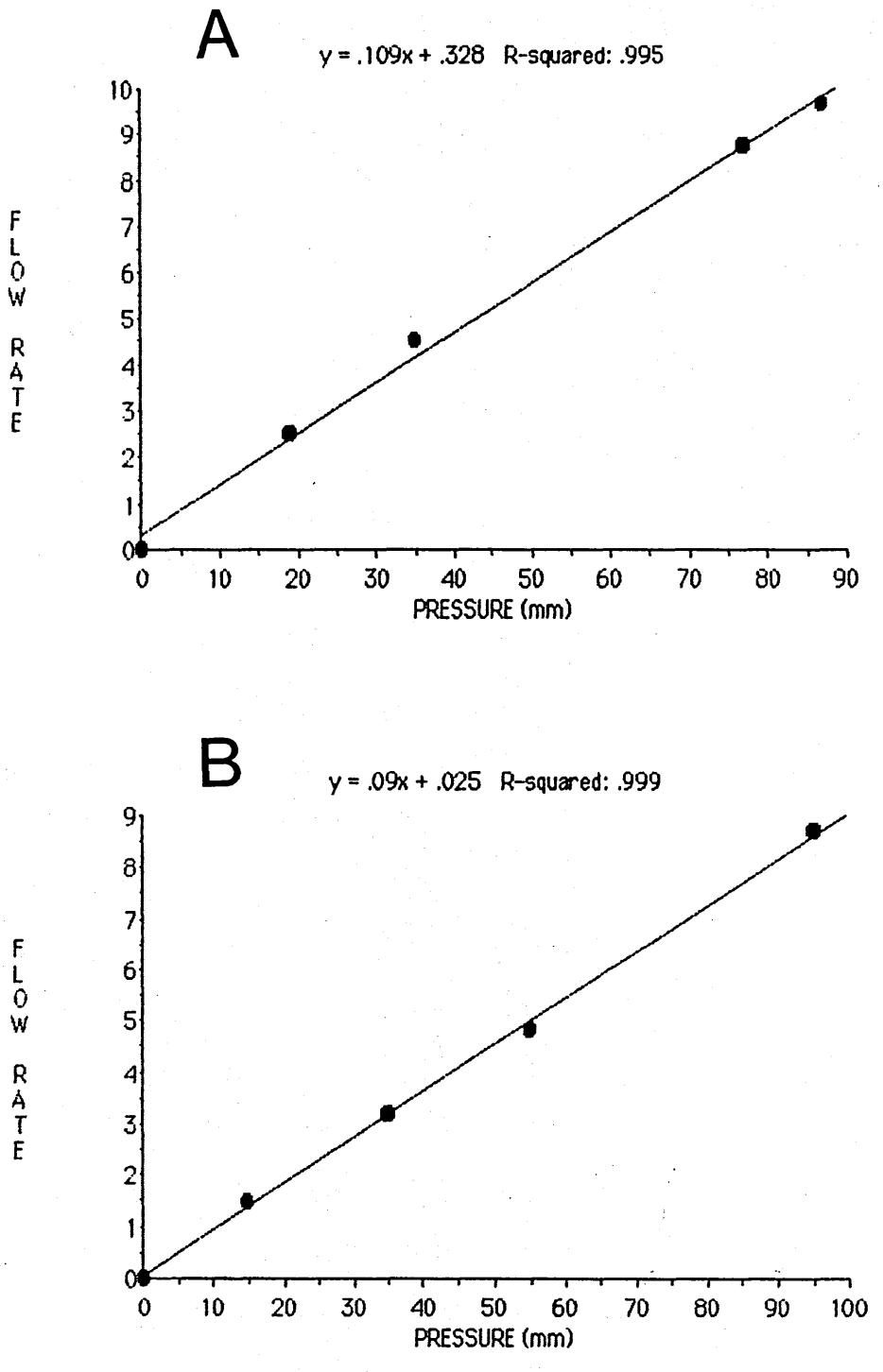


FIGURE 2.12. A & B. Calibration curves for capillary flowmeters 1 and 2 of apparatus A. Each point on the curve represents the mean of three readings. Ordinate: Flow rate in $l\,h^{-1}$. Abscissa: Pressure in mm, where pressure represents the difference in fluid levels in the two arms of the manometer.

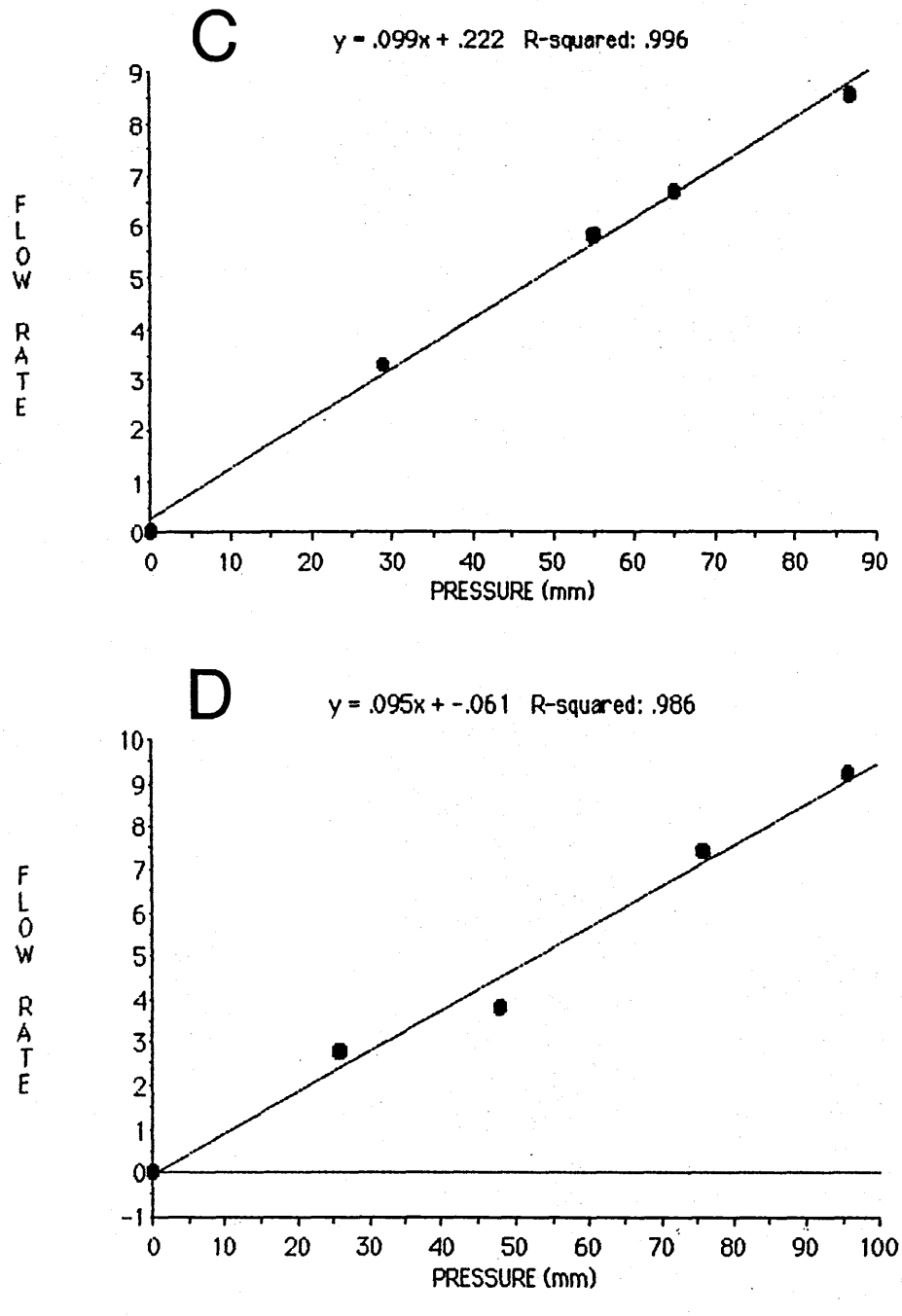


FIGURE 2.12 C & D. Calibration curves for capillary flowmeters 3 and 4 of apparatus A. Each point on the curve represents the mean of three readings. Ordinate: Flow rate in $l\,h^{-1}$. Abscissa: Pressure in mm, where pressure represents the difference in fluid levels in the two arms of the manometer.

of appropriate peaks in control and treated leaves.

Where examples of the rhythm traces are shown these are representative of the data obtained in at least two, but usually more, experiments. These graphs were plotted on an Apple Macintosh Plus computer using the "Microsoft Chart" programme. The values obtained from each sample of leaves in $\mu g\ CO_2\ h^{-1}\ g$ (fresh weight) $^{-1}$ were fed into the computer. The computer plotted these values automatically against time and drew a curve through all the points. In several curves, the rate of CO_2 uptake or output by the leaves appears to be absolutely constant for a few hours at the peaks and troughs in the rhythm. These flat areas on the curves do not represent the true rate of uptake or output of CO_2; they are due to CO_2 uptake or output exceeding the maximum value which could be recorded by the computer even at the low sensitivity setting of the IRGA.

Where data have been statistically analysed and presented graphically, the mean number of observations are provided together with the standard error (S.E.) of the mean. The S.E. is represented on the graphs by vertical lines drawn above and below the data points, each line thus represents ± 1 S.E. These graphs were also plotted on an Apple Macintosh Plus computer using the "Cricket" programme. In addition to plotting the graphs, the "Cricket" programme also calculated and drew regression lines through the points on the graph.

The Students t-test was used to determine whether rhythms exhibited by leaves exposed to different treatments differed significantly. Probability levels were obtained from the Student's t-distribution tables (Fisher and Yates, 1963), differences between means being designated significant when the probability value was less than 0.05. A few of these calculations were carried out on the Apple Macintosh Plus computer using the "Statview" programme, but most were made using a programme written in the department for the BBC computer.

3. RESULTS

3.1. THE EFFECTS OF TEMPERATURE ON THE RHYTHM OF CO_2 EXCHANGE IN LEAVES OF *BRYOPHYLLUM FEDTSCHENKOI*

3.1.1. CONSTANT AMBIENT TEMPERATURE

The effects of constant ambient temperature on the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* have been investigated with three principal objectives. The first was to establish over what temperature range the rhythm operated, the functional range; the second was to examine the value and stability of the period as a function of temperature within the functional range, and the third was to determine the nature of any inhibition which occurred at temperatures outside the functional range. Throughout this investigation the temperatures were controlled to ± 0.1°C and the quantum fluence rate to which the leaves were exposed was 11 - 12 μ mol m^{-2} sec^{-1}.

3.1.1.1. The Functional Range

Persistent rhythms in the rate of CO_2 exchange occurred only in leaves held at temperatures between 10 and 30°C. A representative selection of results from experiments within this temperature range is shown in Figs. 3.1 - 3.8. At each temperature the rhythm persisted for many days.

A detailed analysis has been made of the value and stability of the period at selected constant temperatures. Four independent experiments were carried out at each of the following temperatures: 10, 15, 20, 25 and 30°C. Since time was limiting, at

Figure 3.1. The circadian rhythm of CO_2 exchange in two samples of leaves of *Bryophyllum fedtschenkoi* simultaneously held in continuous light and a stream of normal air at 10°C. Ordinate: the rate of uptake of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.1

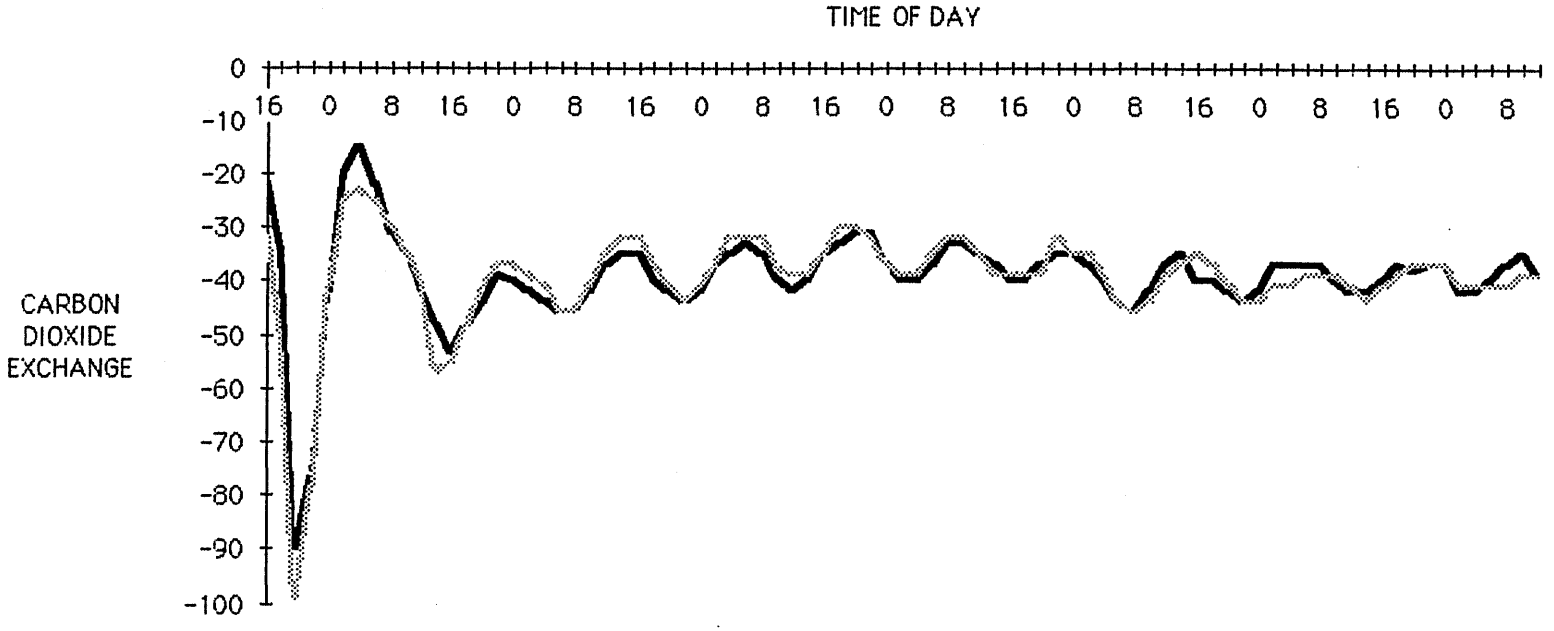

TIME OF DAY
0
16 0 8 16 0 8 16 0 8 16 0 8 16 0 8 16 0 8 16 0 8
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
CARBON DIOXIDE EXCHANGE

Figure 3.2. The circadian rhythm of CO_2 exchange in two samples of leaves simultaneously held in continuous light and a stream of normal air at 12.5°C. Ordinate: the rate of uptake of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.2

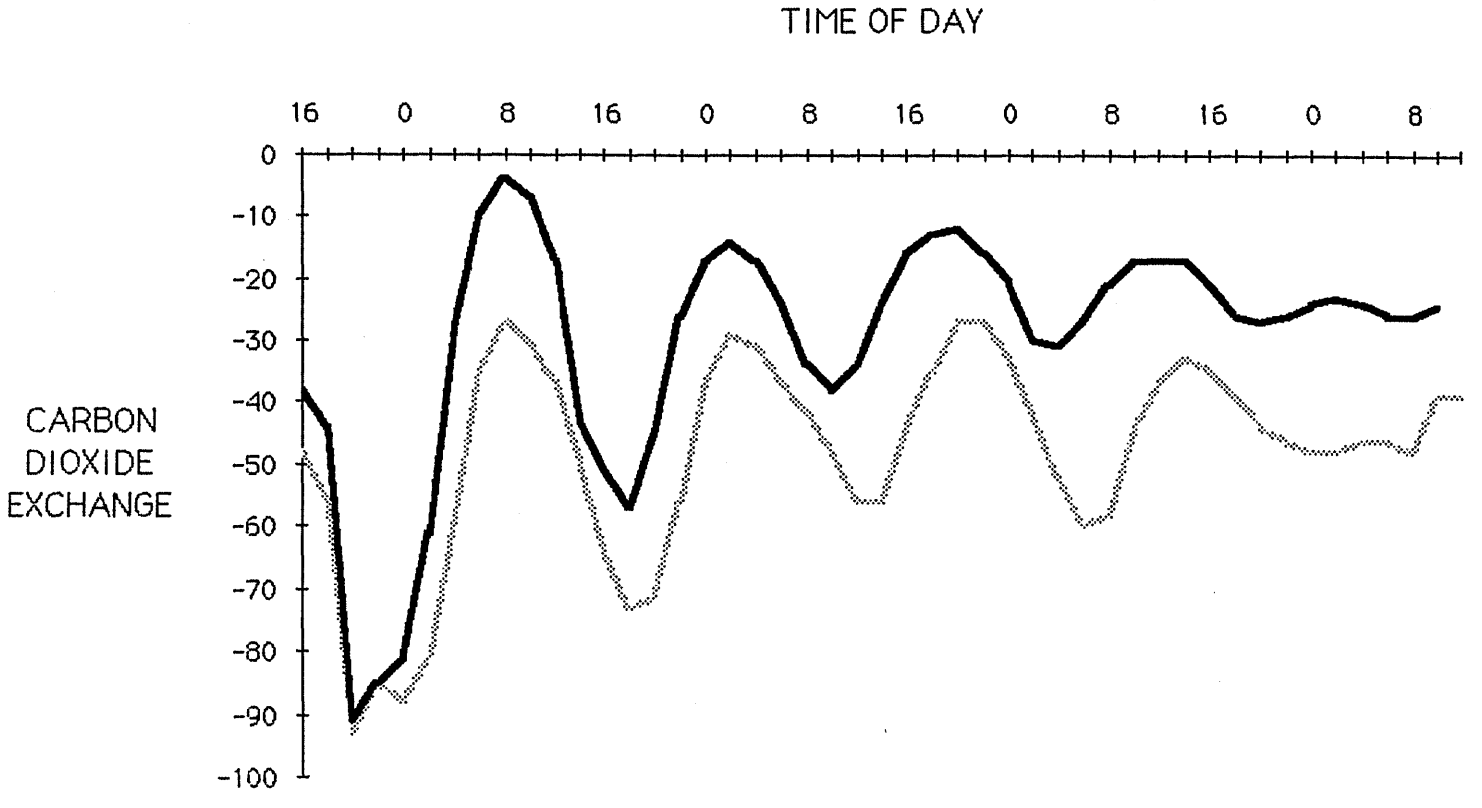

TIME OF DAY
16
0
8
16
0
8
16
0
8
16
0
8
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
CARBON
DIOXIDE
EXCHANGE

FIGURE 3.3. The circadian rhythm of CO_2 exchange in two samples of leaves simultaneously held in continuous light and a stream of normal air at 15°C. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g$ (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.3

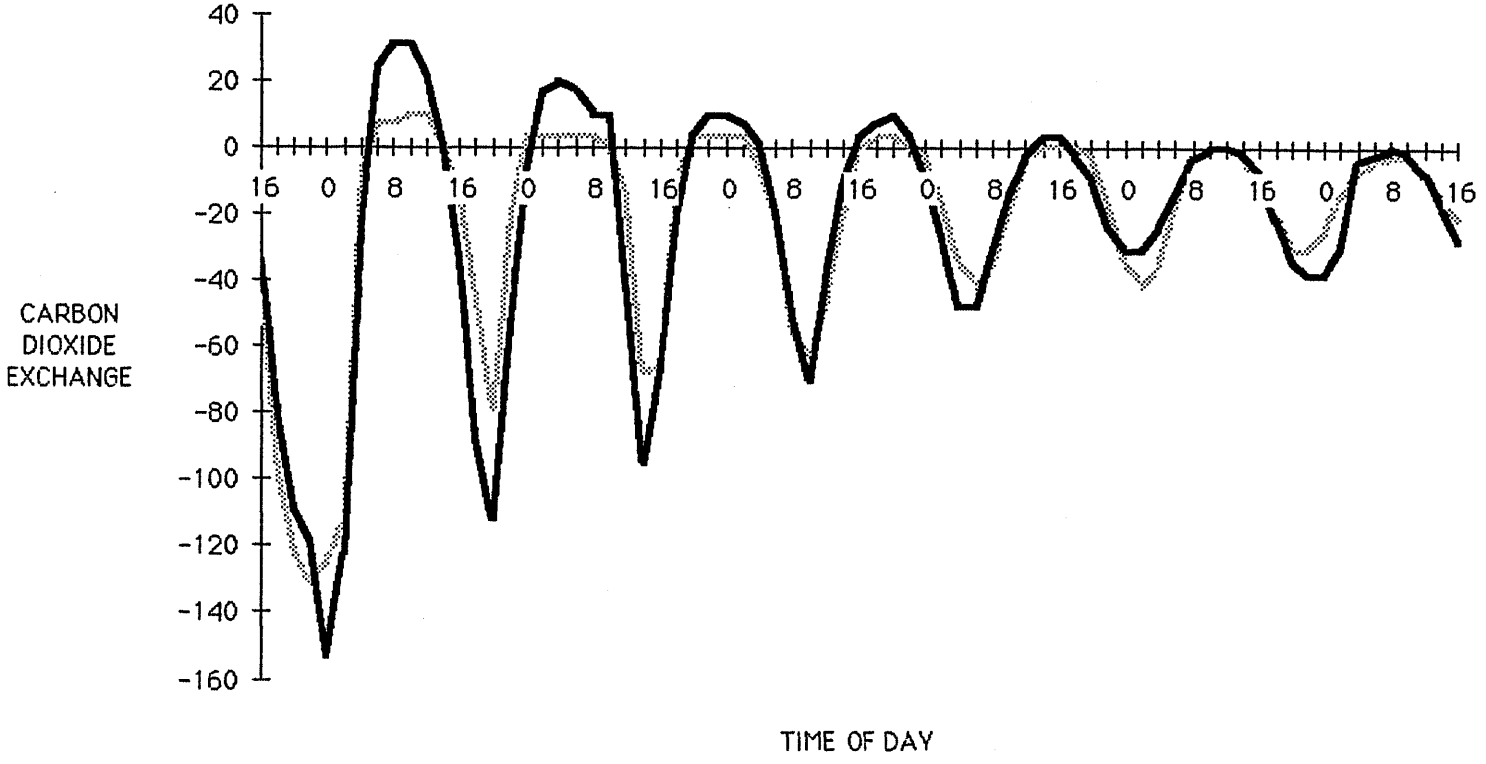

40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.4. The circadian rhythm of CO_2 exchange in two samples of leaves simultaneously held in continuous light and a stream of normal air at 18°C. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g$ (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.4

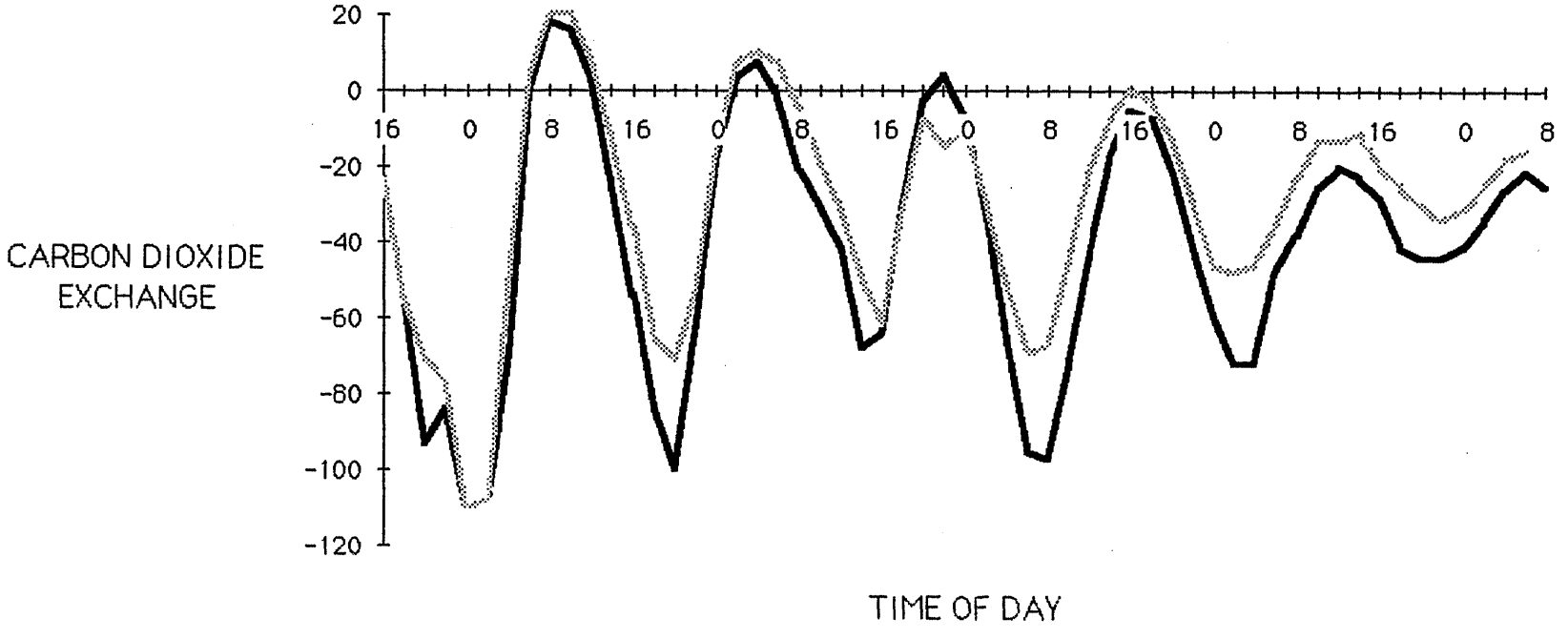

CARBON DIOXIDE EXCHANGE
20
0
-20
-40
-60
-80
-100
-120
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
TIME OF DAY

FIGURE 3.5. The circadian rhythm of CO_2 exchange in two samples of leaves simultaneously held in continuous light and a stream of normal air at 20°C. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in μg CO_2 h^{-1} g (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.5

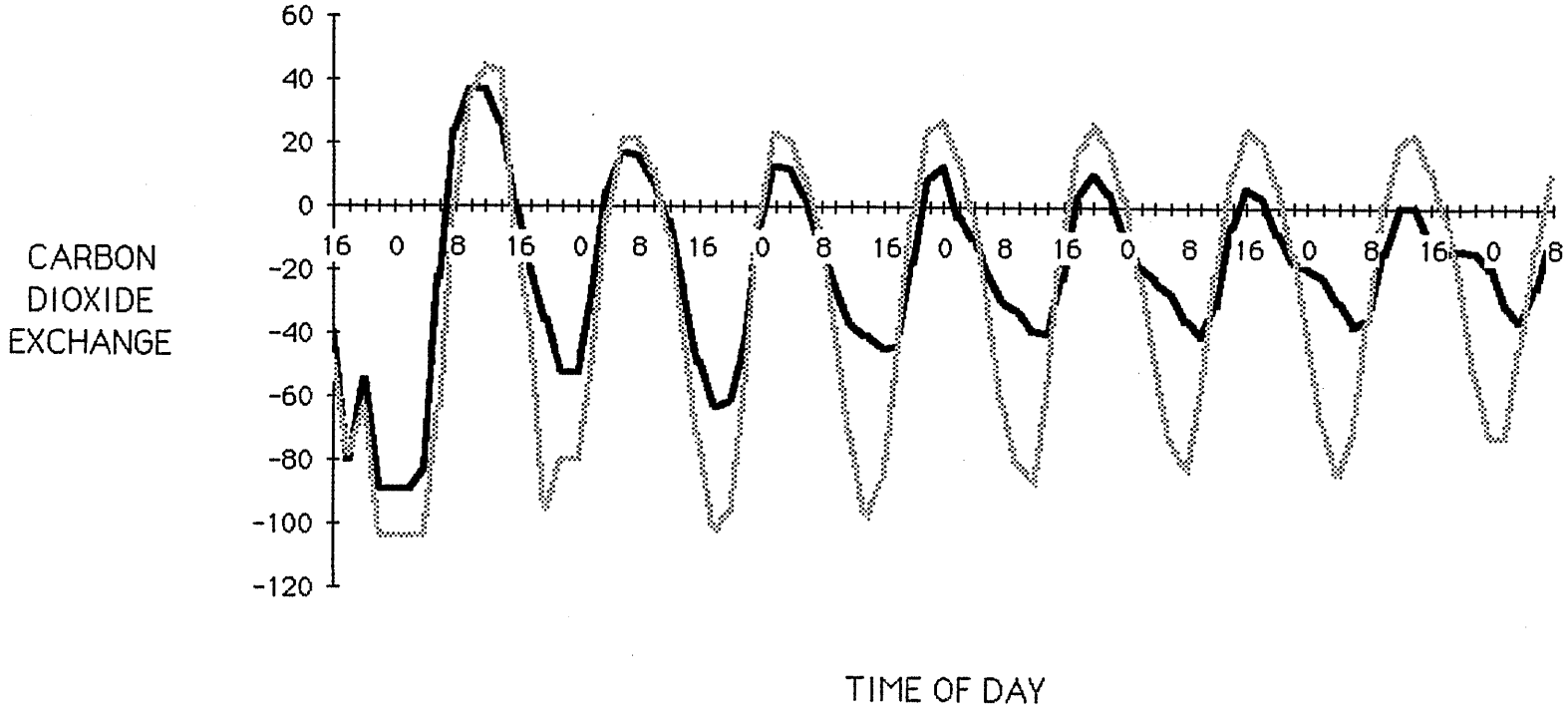

CARBON
DIOXIDE
EXCHANGE
60
40
20
0
-20
-40
-60
-80
-100
-120
16
0
8
TIME OF DAY

FIGURE 3.6. The circadian rhythm of CO_2 exchange in two samples of leaves simultaneously held in continuous light and a stream of normal air at 25°C. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g$ (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.6

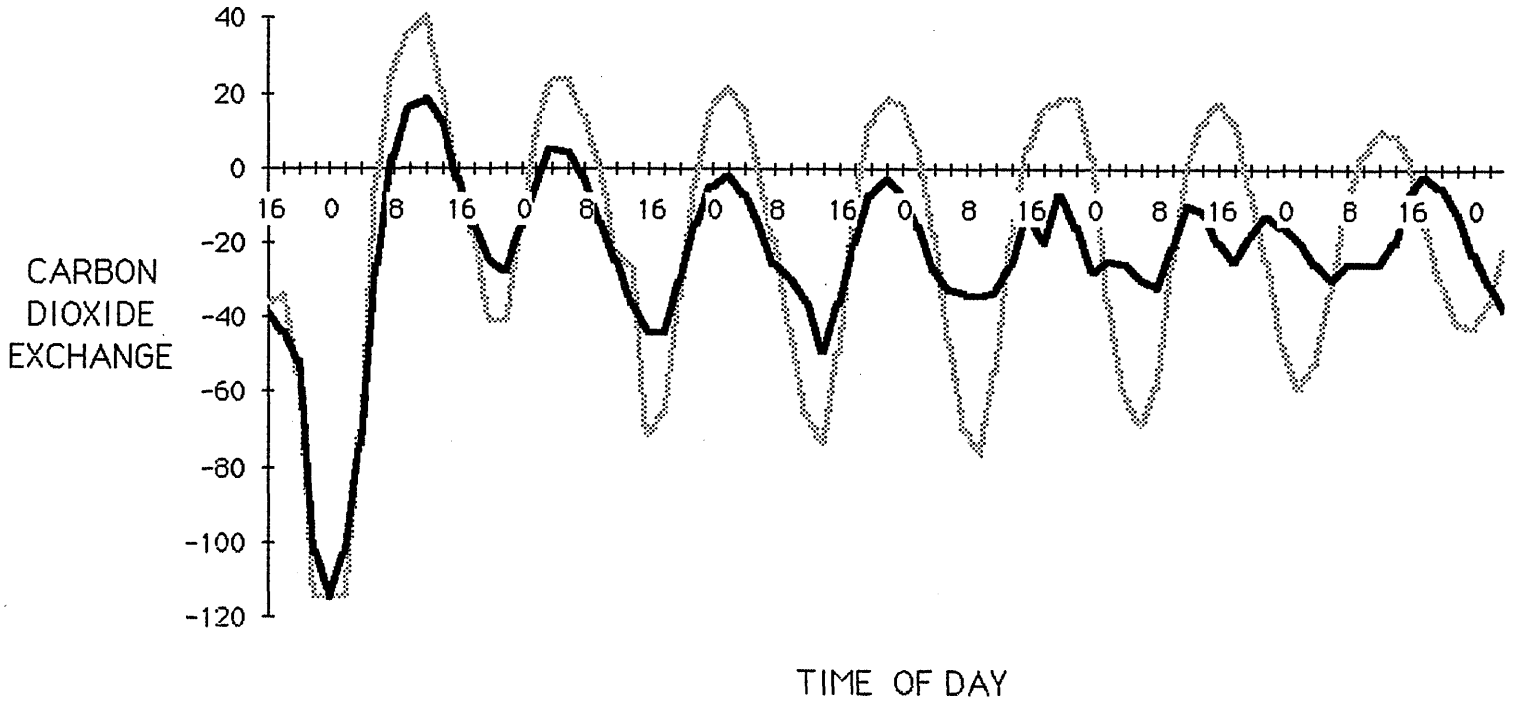

40
20
0
-20
-40
-60
-80
-100
-120
16
0
8
CARBON
DIOXIDE
EXCHANGE
TIME OF DAY

FIGURE 3.7. The circadian rhythm of CO_2 exchange in two samples of leaves simultaneously held in continuous light and a stream of normal air at 28°C. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g$ (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.7

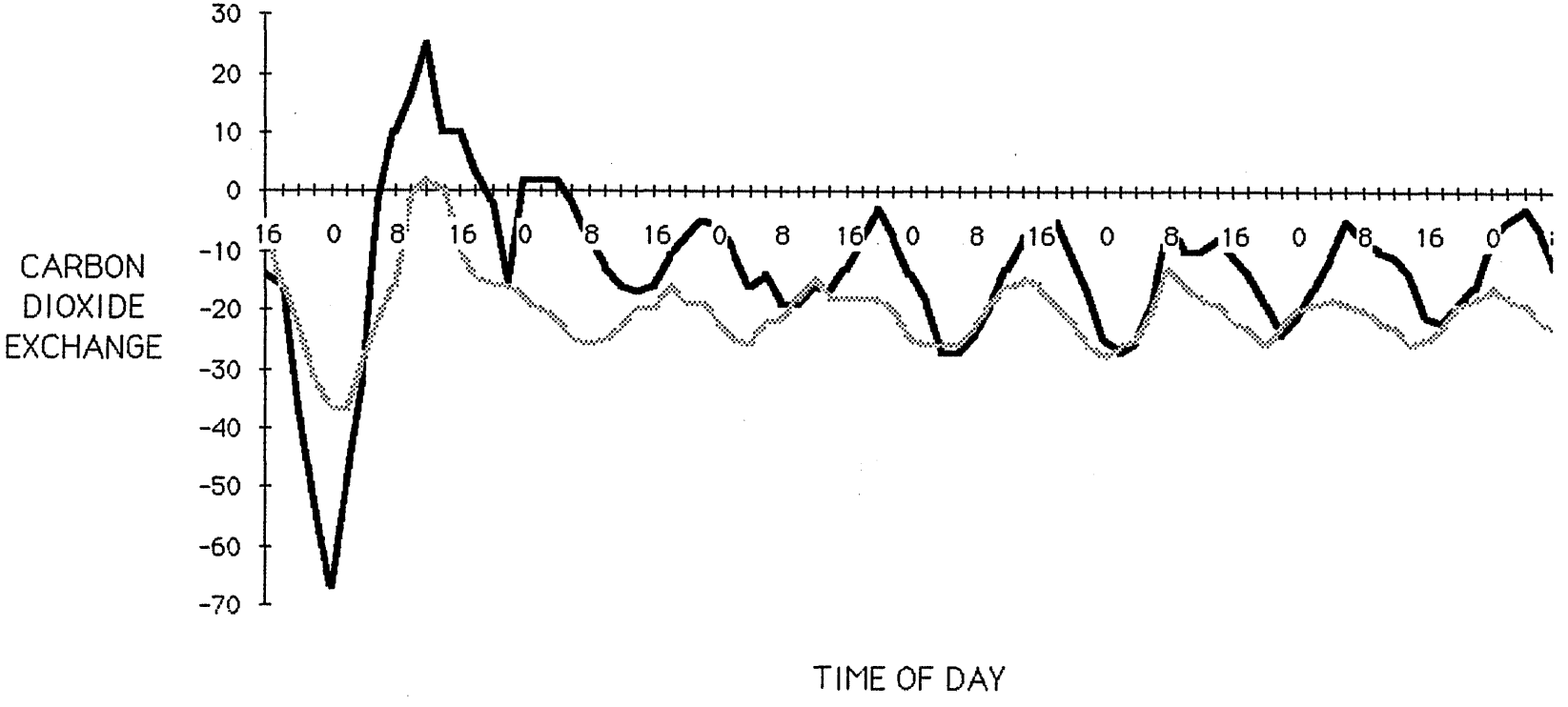
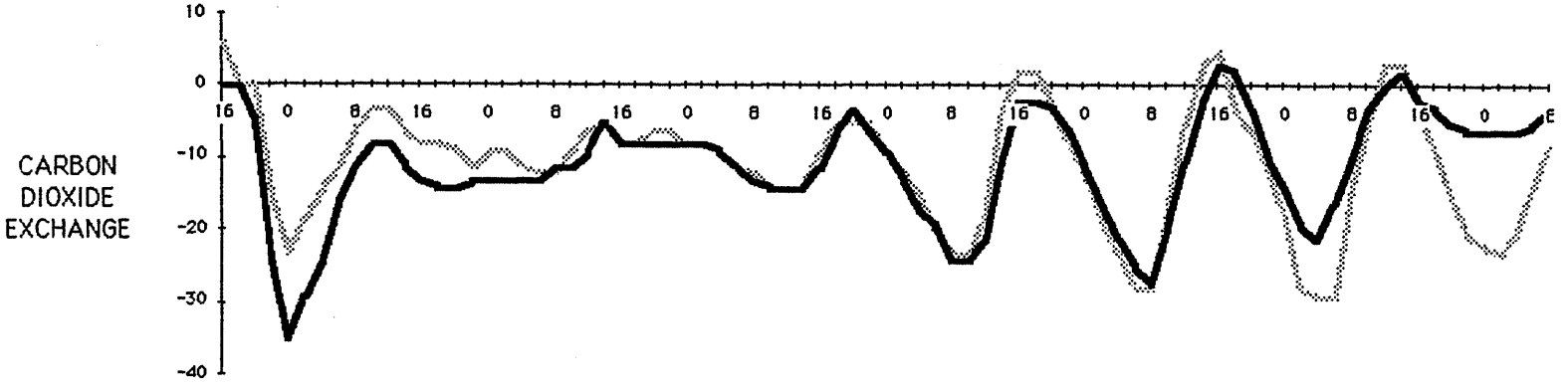

30
20
10
0
-10
-20
-30
-40
-50
-60
-70
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
CARBON
DIOXIDE
EXCHANGE
TIME OF DAY

FIGURE 3.8. The circadian rhythm of CO_2 exchange in two samples of leaves simultaneously held in continuous light and a stream of normal air at 30°C. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g$ (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.8


CARBON
DIOXIDE
EXCHANGE
10
0
-10
-20
-30
-40
16
0
8
16
0
8
16
0
8

each of the intermediate temperatures, 12.5, 18 and 28°C, only two independent experiments were conducted. The data are presented in Figs. 3.9 and 3.10 and in Table 1.

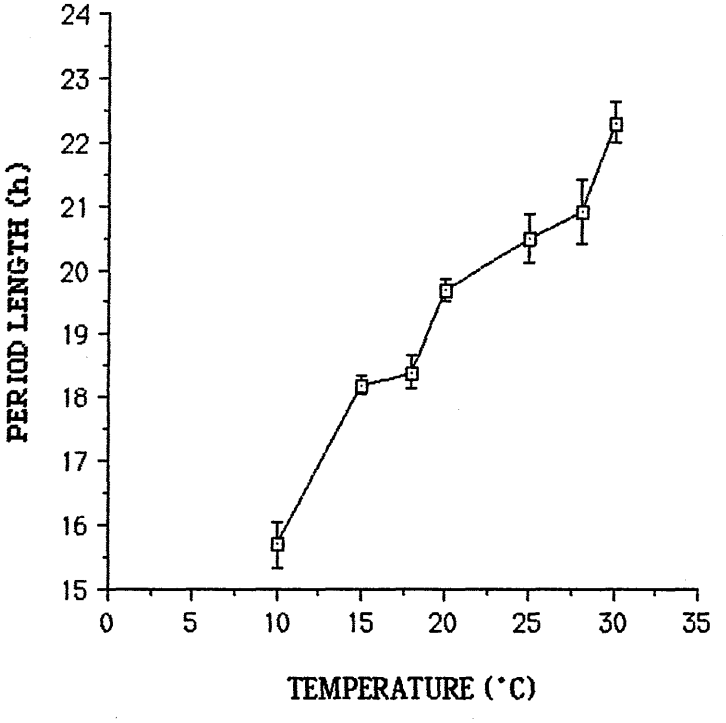


FIGURE 3.9. Values of the period of the rhythm of CO_2 exchange in leaves of *B. fedtschenkoi* kept at various constant ambient temperatures. Each point on the curve represents the mean of at least 20 values. The vertical lines represent ± the standard error of the means. Ordinate: average period (hours). Abscissa: temperature in °C.

At 10°C the period was 15.7 ± 0.35 h, a value which resulted in approximately three oscillations of the rhythm occurring every 48 h. The period increased almost linearly with increasing temperature to attain a maximum value of 22.3 ± 0.33 h at 30°C. A detailed analysis of the period at 30°C was difficult, however, because the delayed appearance of the rhythm made the number of periods available for

measurement rather small. At all temperatures the period was much less than 24 h. The temperature coefficients for the period (period at t-10^{o}C/period at t^{o}C) were calculated at three different 10^{o}C intervals within the range of 10 to 28^{o}C and are shown in Table 1. A temperature coefficient calculated in this manner is referred to in the field of circadian rhythms as a Q_{10}; this term shall be used throughout the remainder of this investigation. The similarity of the Q_{10} values would be expected from the apparently linear nature of the relationship between period and temperature. However, the very closeness of these to unity should be noted, since they indicate clearly a considerable degree of temperature compensation in the oscillating system. Attention is also drawn to the fact that these values are all less than one.

TABLE 1.

TEMPERATURE COEFFICIENTS WITHIN THE RANGE 10 - 28^{o}C.

TEMPERATURE (^{o}C)	TEMPERATURE COEFFICIENT
10/20	0.80
15/25	0.89
18/28	0.88

The stability of the period at each temperature was assessed by determining the lengths of successive periods of the rhythm in individual leaf samples. The results of this assessment are shown in Fig. 3.10 and reveal a number of interesting features.

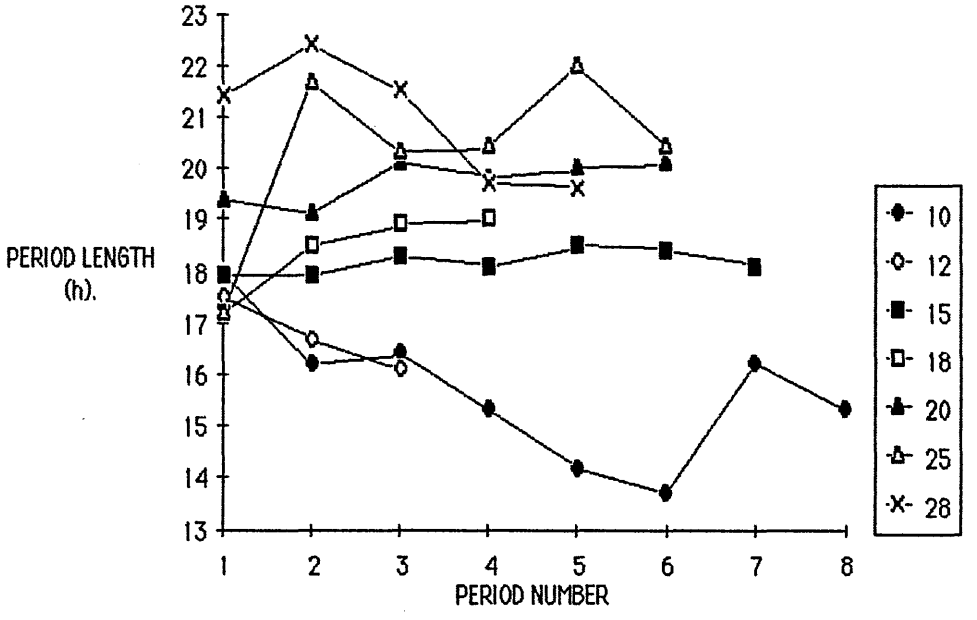


FIGURE 3.10. Values of successive periods (mean of at least 5 values) of the rhythm in leaves maintained at various constant ambient temperatures. The box identifies each curve with a particular temperature in ^{o}C. Ordinate: average period (hours). Abscissa: period number.

At 10^{o}C, there is a gradual shortening of successive periods from the first of 18.0 ± 0.45 h, to the sixth, at which the unusually short value of 13.7 ± 0.25 h was recorded. After the sixth period, however, this pattern breaks down, the seventh period being 16.2 ± 0.17 h and the eighth 15.3 ± 1.1 h. A similar shortening of the period appears to occur at 12.5^{o}C, but the rhythm was not monitored over a sufficient length of time at this temperature to be certain that this is indeed the case. No evidence of period instability at this temperature was provided by the available data

At temperatures within the range of 15 - 20^{o}C the period appeared to be relatively stable although there is some indication at 18^{o}C that the steady-state period is not reached until after the first cycle is completed because the first period

was significantly shorter than all successive periods. This point would, however, require further investigation since the difference is only just significant at the 0.05 probability level and time permitted only two experiments to be carried out at this temperature.

At 15 and 20°C complete period stability was observed, no significant differences were detected between successive periods at these temperatures.

Marked instability of the period was observed at 25°C, a difference of approximately 3 h existing between the first period of 17.2 ± 0.30 h, and the average period 20.5 ± 0.88 h. Significant differences also exist between the second and fourth periods, and the fourth and fifth periods, indicating a general instability of the period at this temperature without any consistent trend towards either shorter or longer values.

Although the appearance of the curve at 28°C indicates that the period is also unstable at this temperature, statistical analysis does not support this view, no significant differences occurred between the lengths of any of the periods at this temperature. Such a finding is perhaps surprising, particularly in view of the fact that 28°C is approaching the upper temperature limit for oscillation to occur.

Other features and characteristics of the rhythms at different ambient temperatures, such as the amplitude, persistence and damping pattern have not been subject to systematic study since they are likely to be highly dependent upon the age and physiological state of the leaves. Considerable variation was in fact found between one leaf sample and another. However, inspection of the curves presented in Figs. 3.1 - 3.8 show that the amplitude of the rhythm is low at 10°C (Fig. 3.1), increases to a maximum value which is maintained over the range of 15 - 20°C and thereafter decreases to a low value again at 25 - 30°C. No attempt was made to study the persistence of the rhythm with time since it was clear from two preliminary experiments at 15°C that the rhythm would continue for at least 14 days, and it was decided that the time involved in assessing this parameter at

different temperatures could not be justified at this stage of the investigation.

The damping pattern of the rhythm also appeared to be influenced by temperature. At 10°C the first peak had a large amplitude and was followed by at least 10 cycles in which the peaks had a much lower amplitude. At temperatures between 12.5 and 25°C there appeared to be a gradual decrease in amplitude with time, whilst at 28°C a pattern somewhat similar to that found at 10°C was apparent.

An unusual feature was the delayed appearance of the rhythm at 30°C. About 72 h usually elapsed before the rhythm could be observed. Whether this delay represented a temporary inhibition of the rhythm, or the failure of a rhythm to manifest itself, has not been established.

The average value of CO_2 exchange around which oscillation occurred at the different temperatures was not studied in detail but it appears from the data presented here that the oscillations occur around a mean rate of fixation of CO_2 of about 40 - 50 $\mu g\ CO_2\ h^{-1}$ g (fresh weight)$^{-1}$ over the range from 10 to 20°C. This value decreased at higher temperatures to about 15 $\mu g\ CO_2\ h^{-1}$ g (fresh weight)$^{-1}$ at 28°C.

A final point concerns the possible influence of temperature on the phase of the rhythm. In order to investigate such a possibility the number of hours between 1600 h, when the experiments were set up, and the time of occurrence of the first peak of the rhythm was recorded at temperatures within the range of 10 - 25°C. The results are presented in Table 2.

TABLE 2

RELATIONSHIP BETWEEN TEMPERATURE AND TIME TAKEN TO REACH FIRST PEAK

TEMPERATURE IN ^{0}C	TIME (h) FROM 1600 h TO FIRST PEAK
10	18.7 ± 0.68
12.5	17.2 ± 0.40
15	17.6 ± 0.50
18	16.9 ± 0.44
20	19.7 ± 0.44
25	19.7 ± 0.39

Clearly no simple relationship exists between temperature and the number of hours taken to reach the first peak of the rhythm. The time which elapsed between setting up the experiments at 1600 h and the time of occurrence of the first peak at 12.5, 15 and 18^{0}C is significantly shorter than the time which elapsed in leaves maintained at 20 and 25^{0}C. However, significant differences were not observed between the time taken to reach the first peak at 10^{0}C and that taken at any of the other temperatures investigated. A more detailed analysis of the relationship between temperature and the time of occurrence of the first peak of the rhythm would be required to establish the influence of temperature on the phase of the rhythm but it was felt that such a study was outwith the main objectives of this investigation.

The stability of the period and large amplitude of the rhythm at 15^{0}C was the reason why this temperature was selected as the base temperature for the remainder of this investigation.

At temperatures above 30^{0}C and below 10^{0}C no rhythm could be detected in the

CO_2 exchange of the *Bryophyllum* leaves. The results of exposing leaves to inhibitory temperatures are discussed in the next section.

3.1.1.2. Inhibitory Range

Figs. 3.11 and 3.12 show the behaviour of leaves maintained for several days at 35 and 40°C respectively. At 35°C (Fig. 3.11) the rate of CO_2 output decreased over the first 16 - 20 h, net output being replaced by net uptake at the end of this time. Thereafter, CO_2 is fixed at a more or less constant rate of approximately 5 $\mu g\ CO_2\ h^{-1}$ g (fresh weight)$^{-1}$ for at least a further 3 days. A somewhat similar pattern of behaviour was observed in leaves held at 40°C (Fig. 3.12), except that at this temperature the rate of CO_2 output decreased gradually over a period of 2 - 3 days, no significant CO_2 fixation occurring at any time during the experimental period.

Although no rhythm was observed in leaves exposed to low temperatures, the pattern of CO_2 exchange was quite different from that observed at high temperatures. The results of holding leaves at 5 and 2°C are illustrated in Figs. 3.13 and 3.14 respectively.

At 5 and 2°C the rate of CO_2 uptake increased for about 12 h, attaining a maximum value between midnight and 0400 h on the second day and subsequently decreased gradually for a further 18 - 20 h when a more or less stable value was attained. For at least the next 3 days at 5 or 2°C the rate of CO_2 uptake remained virtually constant at approximately 40 - 50 $\mu g\ CO_2\ h^{-1}$ g (fresh weight)$^{-1}$, a rather surprising finding since enzyme activity at these temperatures might have been expected to be low. That this rate of fixation is due to photosynthesis alone is indicated by the cessation of net CO_2 uptake and its replacement by net output when

FIGURE 3.11. Inhibition of the rhythm of CO_2 exchange in two samples of leaves of *Bryophyllum fedtschenkoi* simultaneously held in continuous light and a stream of normal air at 35°C. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.11

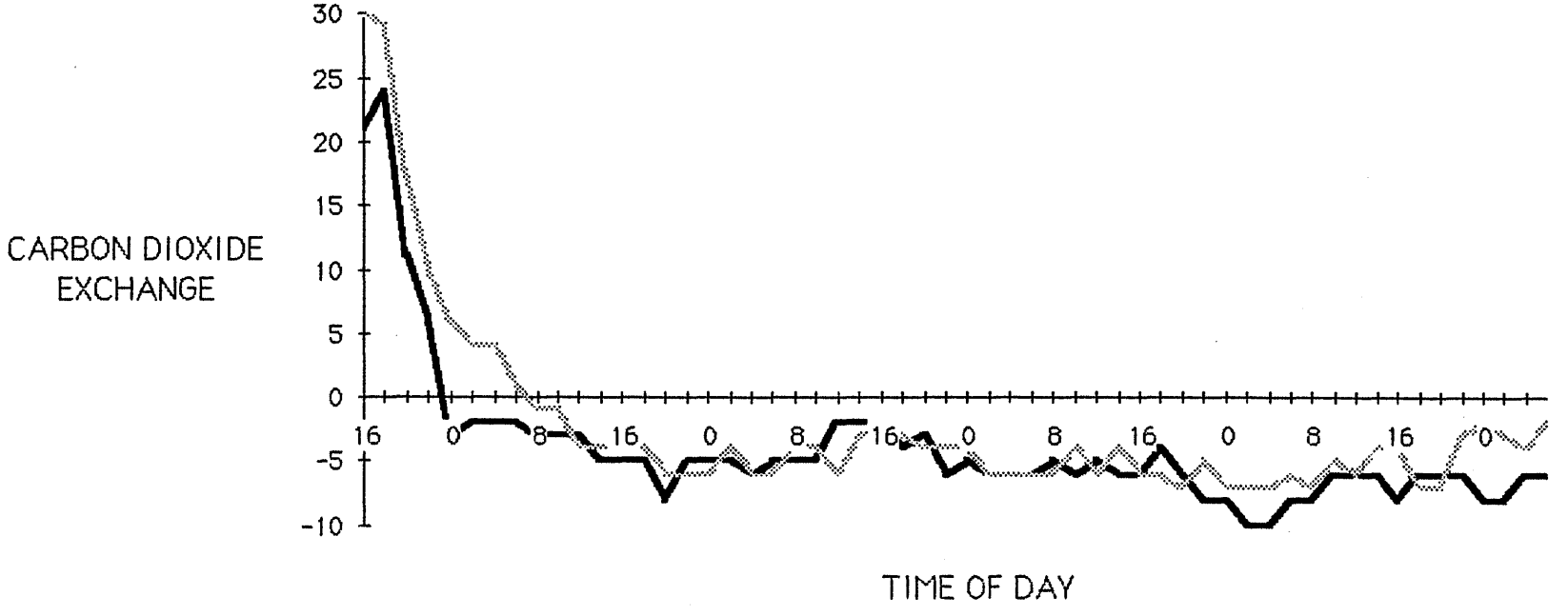

CARBON DIOXIDE EXCHANGE
30
25
20
15
10
5
0
-5
-10
16
0
8
16
0
8
16
0
8
16
0
8
16
0
TIME OF DAY

FIGURE 3.12. Inhibition of the rhythm of CO_2 exchange in two samples of leaves simultaneously held in continuous light and a stream of normal air at 40°C. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g$ (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.12

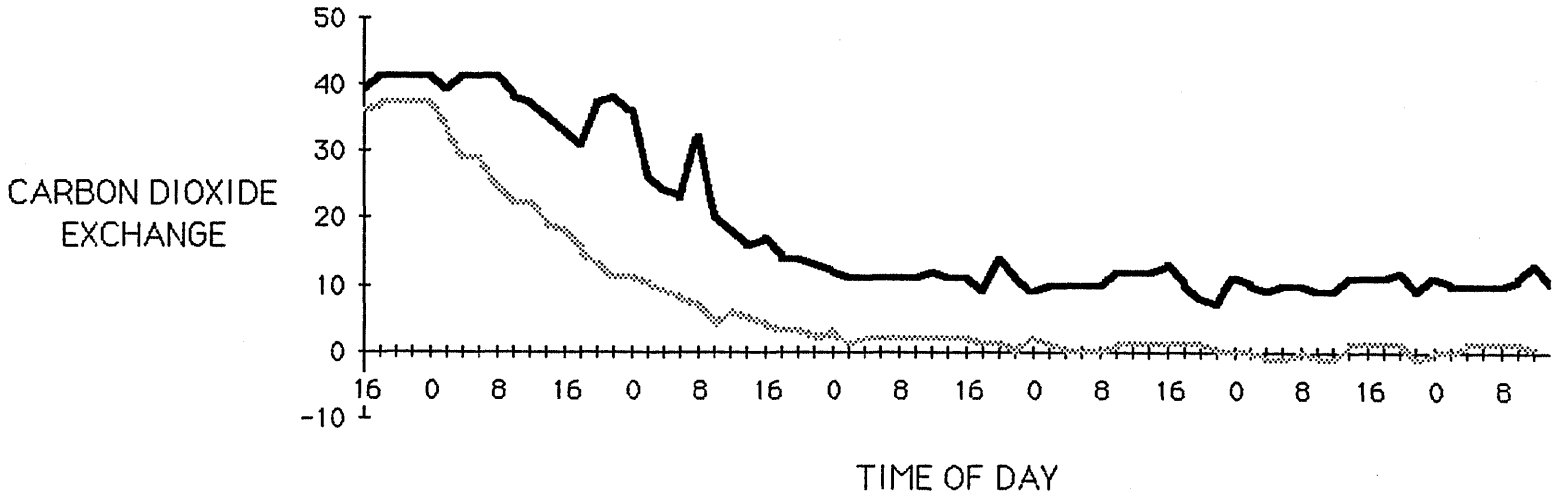

CARBON DIOXIDE EXCHANGE
50
40
30
20
10
0
-10
16 0 8 16 0 8 16 0 8 16 0 8 16 0 8 16 0 8
TIME OF DAY

FIGURE 3.13. Inhibition of the rhythm of CO_2 exchange in two samples of leaves held simultaneously at 5°C. The arrow indicates the time at which the light was extinguished (0800 h). Leaves were otherwise maintained in a stream of normal air and continuous illumination. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.13

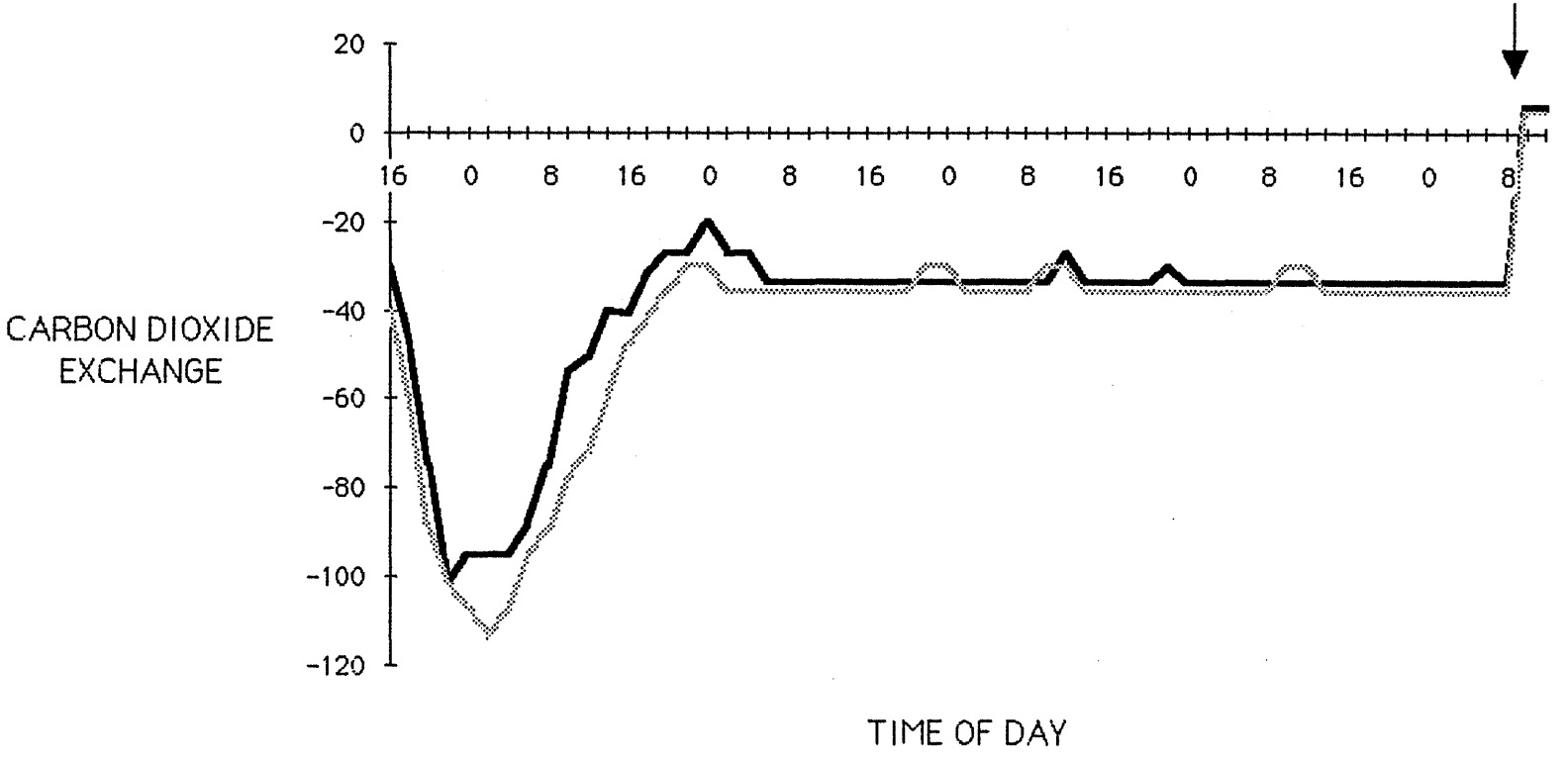
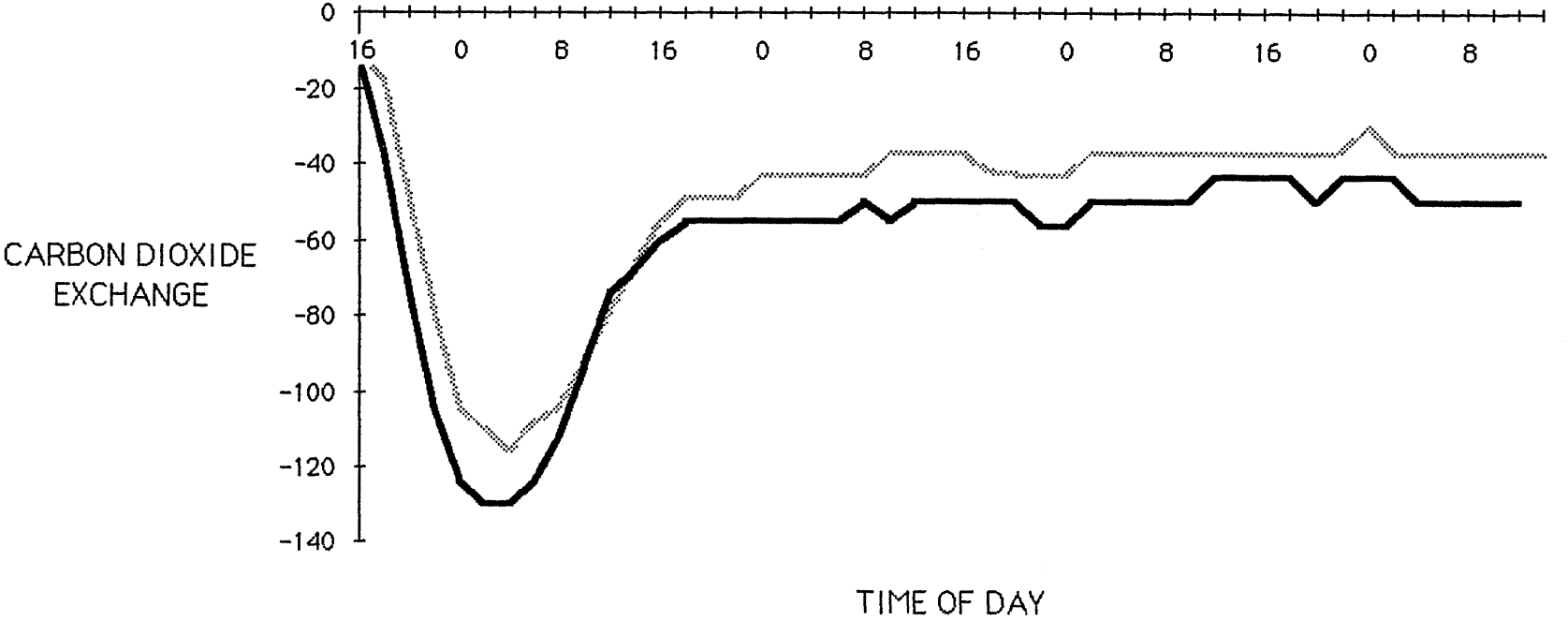

20
0
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
-20
-40
-60
-80
-100
-120
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.14. Inhibition of the rhythm of CO_2 exchange in two samples of leaves held simultaneously in continuous light and a stream of normal air at 2°C. Ordinate: the rate of uptake of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.14


0
16
0
8
16
0
8
16
0
8
16
0
8
-20
-40
-60
-80
-100
-120
-140
CARBON DIOXIDE EXCHANGE
TIME OF DAY

the light is extinguished at the time indicated by the arrow in Fig. 3.13.

The results in this section have established a number of important features of the rhythm. First, the rhythm of CO_2 exchange in leaves continuously illuminated in a stream of normal air operates within the approximate temperature range of 10 - 30°C. Second, the period of the rhythm, which is unusually short for circadian rhythms, especially at the lowest temperatures, increases significantly and linearly with increasing temperature from 15.7± 0.35 h at 10°C to 22.3 ± 0.33 h at 30°C. The effect of temperature on the period is, however, relatively small, the average Q_{10} over the range being approximately 0.86, a figure indicative of a considerable degree of temperature compensation. Third, within the functional temperature range there is period stability between 12.5 and 20°C and period instability at 10 and 25 - 28°C.

3.1.2. THE NATURE OF THE INHIBITION OF THE RHYTHM IN LEAVES HELD AT EXTREME TEMPERATURES. (i.e 40 and 2°C)

3.1.2.1. Step-Type Temperature Changes

In the previous section it was reported that no circadian rhythm of CO_2 exchange could be detected in leaves held at 40 and 2°C. The absence of a detectable rhythm at these temperatures may be due firstly to the basic oscillating mechanism continuing to operate but being unable to manifest itself, secondly to the basic oscillator being irreparably damaged, or thirdly to the oscillator being merely inhibited. To resolve this problem leaves held at 40°C and 2°C for several days were transferred to 15°C at different times of the day to establish whether or not the rhythm appeared, and if it did, whether the phase of the new rhythm was related to

the time of transfer. If it emerged from these experiments that the basic oscillating mechanism was, in fact, merely inhibited by exposure to 40 or 2°C then the results presented in the previous section indicate that the nature of the inhibition may be fundamentally different at these two temperatures because little or no CO_2 was fixed by leaves maintained at 40°C whereas large amounts were fixed by leaves held at 2°C.

Representative results obtained when leaves which had been maintained at 40°C were transferred to 15°C are shown in Figs. 3.15 and 3.16 and the collated results of a number of such experiments in which different transfer times were used are presented in Table 3. The times of the temperature change in Figs. 3.15 and 3.16 were noon and midnight respectively on the second day of the experiment.

Whilst again no rhythm was detected at 40°C, a rhythm appeared immediately the leaves were transferred to 15°C and it persisted for at least 5 cycles with an average period of 19.4 ± 0.17 h. No permanent damage to the oscillating mechanism therefore occurs at 40°C.

A major feature of these results is that following the transfer from 40 to 15°C the new rhythm always appears by the leaves immediately beginning to fix large amounts of CO_2 at a rate which increased to a maximum value about 4 h later. As a result of this pattern of behaviour the phase of the new rhythm is therefore wholly determined by the time at which the temperature is reduced from 40 to 15°C. The first peak of the rhythm occurs approximately 15 h after the end of this high temperature treatment regardless of the time of day at which the transfer occurs, a point which is particularly illustrated by the collated data presented in Table 3 where the times of occurrence of the peaks are an average of four measurements.

FIGURE 3.15. Inhibition of the rhythm of CO_2 exchange in two samples of leaves of *B. fedtschenkoi* held simultaneously at 40°C and its restoration on decreasing the temperature to 15°C. The arrow indicates the time at which the temperature change from 40 to 15°C was made (midday). Leaves were otherwise maintained in a stream of normal air and continuous illumination. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g$ (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.15

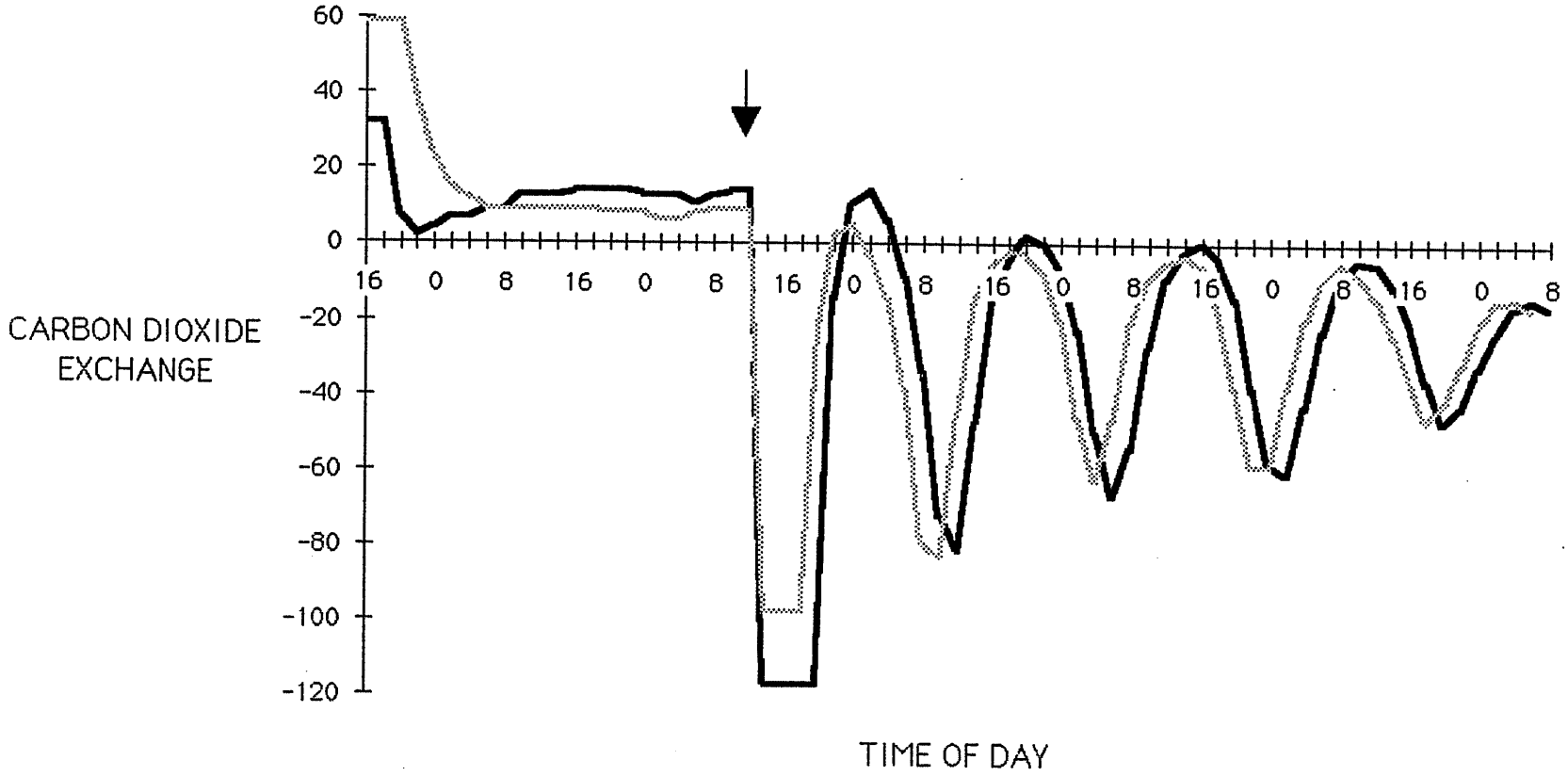

CARBON DIOXIDE EXCHANGE
60
40
20
0
-20
-40
-60
-80
-100
-120
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
TIME OF DAY

FIGURE 3.16. Inhibition of the rhythm of CO_2 exchange in two samples of leaves of *B. fedtschenkoi* held simultaneously at 40°C and its restoration on decreasing the temperature to 15°C. Conditions and presentation are as described for Fig. 3.15 except that the temperature change from 40 to 15°C was made at midnight.

FIGURE 3.16

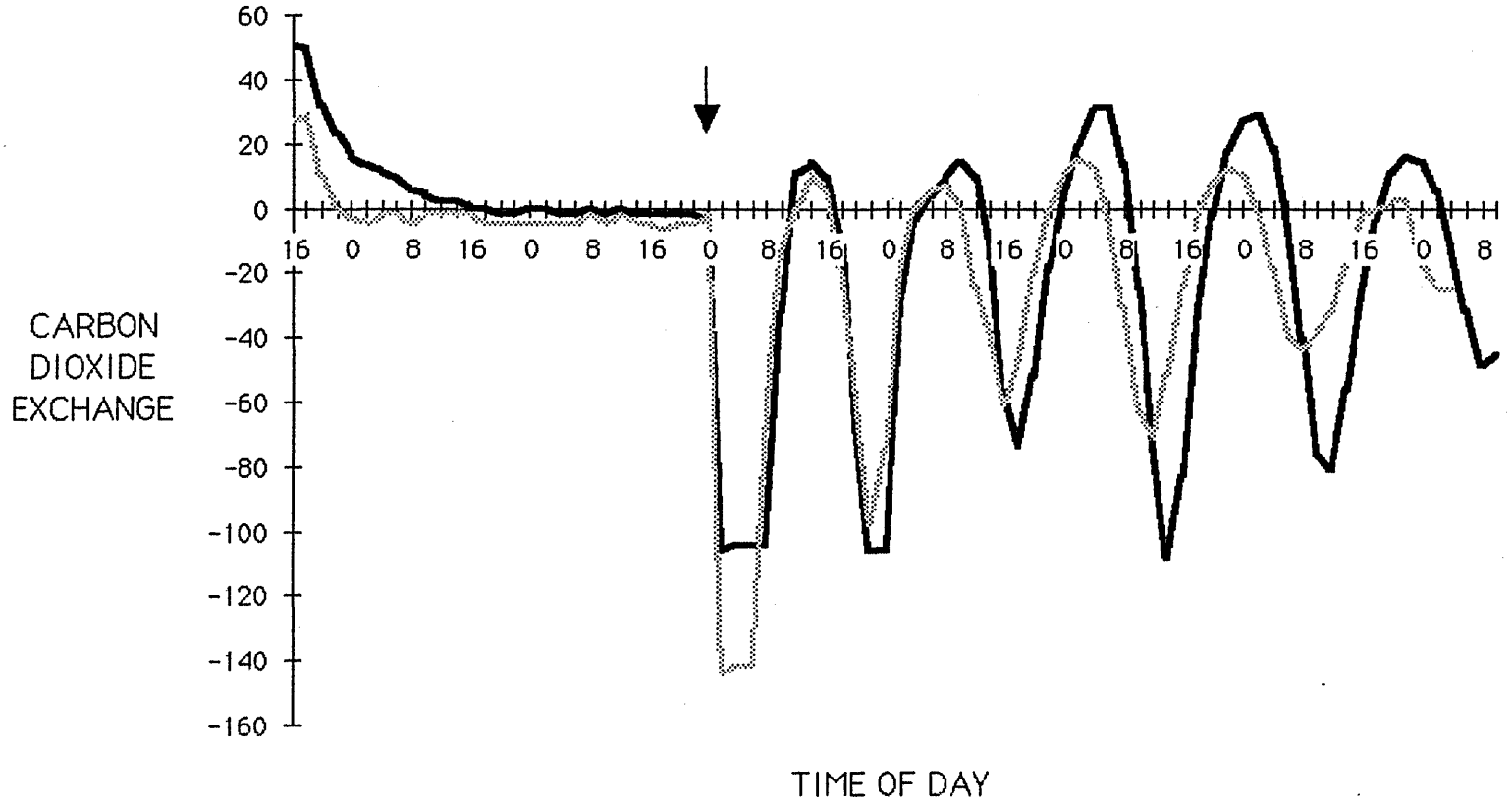

60
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
16 0 8 16 0 8 16 0 8 16 0 8 16 0 8 16 0 8 16 0 8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

TABLE 3.

RELATIONSHIP BETWEEN END OF HIGH TEMPERATURE TREATMENT AND TIME TAKEN TO REACH PEAKS

TIME AT	TIME FROM END OF 40°C TO:		
40°C (h)	1ST PEAK	2ND PEAK	3RD PEAK
38	14	34	53
44	15	33	52
50	16	35	54
56	14	33	53

This finding establishes that the oscillating mechanism controlling the rhythm must be inhibited at 40°C; if it were operating but unable to manifest itself there would not be the fixed relationship between the time of the transfer to 15°C and the phase of the newly initiated rhythm. The relationship also establishes that the oscillator must be inhibited at 40°C by being driven to, and held at, a fixed phase point from which the oscillation always begins when the temperature is reduced to 15°C.

The average period of the rhythm at 15°C following a high temperature treatment was 19.4 ± 0.17 h and appears to be longer than the average value of 18.2 ± 0.16 h previously recorded at 15°C (see section 3.1.1.1). Due to technical constraints associated with an earlier version of the apparatus, simultaneous monitoring of samples of leaves held at 40 and 15°C was not possible. However, comparing the period of the rhythm at 15°C in leaves previously held at 40°C with that in leaves held wholly at 15°C in the same apparatus during later experiments indicates that the period in the former (19.4 ± 0.17 h) is indeed significantly longer than in the latter (18.5 ± 0.15 h). Thus, exposing leaves to 40°C appears to result in the period of the subsequent rhythm at 15°C being slightly longer than that of

control leaves.

The effects of maintaining leaves at 2°C for between 2 and 3 days before raising the temperature to 15°C are shown in Figs. 3.17 and 3.18. The collated results from a number of such experiments in which the temperature change was made at different times are shown in Fig. 3.19. In Fig. 3.17 the temperature was changed from 2 to 15°C at midday and in Fig. 3.18 at midnight on the second day of the experiment. In both of these experiments the rhythms in the two samples of treated leaves gradually became out of phase with one another as time progressed; this was due to a slight difference in the lengths of the periods in the two samples of leaves. The curves at 2°C are closely similar to those presented in Fig. 3.14, and show the major period of CO_2 fixation followed by an approximately constant but lower level of fixation, with no indication of a circadian rhythm. Increasing the temperature from 2 to 15°C led to the immediate appearance of a rhythm in CO_2 exchange which persisted for at least 4 cycles with an average period of 17.8 ± 0.25 h. In each experiment the rhythm began by the leaves immediately decreasing their rate of CO_2 fixation to an extremely low value, so that for about 6 - 8 h there was scarcely any net CO_2 exchange between the leaf and the environment. The rate of CO_2 fixation then increased as the leaves exhibited the familiar circadian rhythm of CO_2 assimilation. The first peak of the rhythm always occurred approximately 4 h after the transfer to 15°C, regardless of the time of day at which the transfer was made. The time of the temperature change therefore determines the phase of the newly initiated rhythm, a point which is particularly illustrated by the collated data presented in Fig. 3.19.

These findings establish unequivocally that exposure to 2°C inhibits the oscillating mechanism, rather than exerting its effect by either preventing the manifestation of its operation or causing irreparable damage to the leaves.

FIGURE 3.17. Inhibition of the rhythm of CO_2 exchange in two samples of leaves of *Bryophyllum fedtschenkoi* held simultaneously at 2°C and its restoration on increasing the temperature to 15°C (continuous lines). The broken line represents the rhythm in control leaves held continuously at 15°C. The arrow indicates the time at which the temperature change from 2 to 15°C was made (midday). Leaves were otherwise maintained in a stream of normal air and continuous illumination. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.17

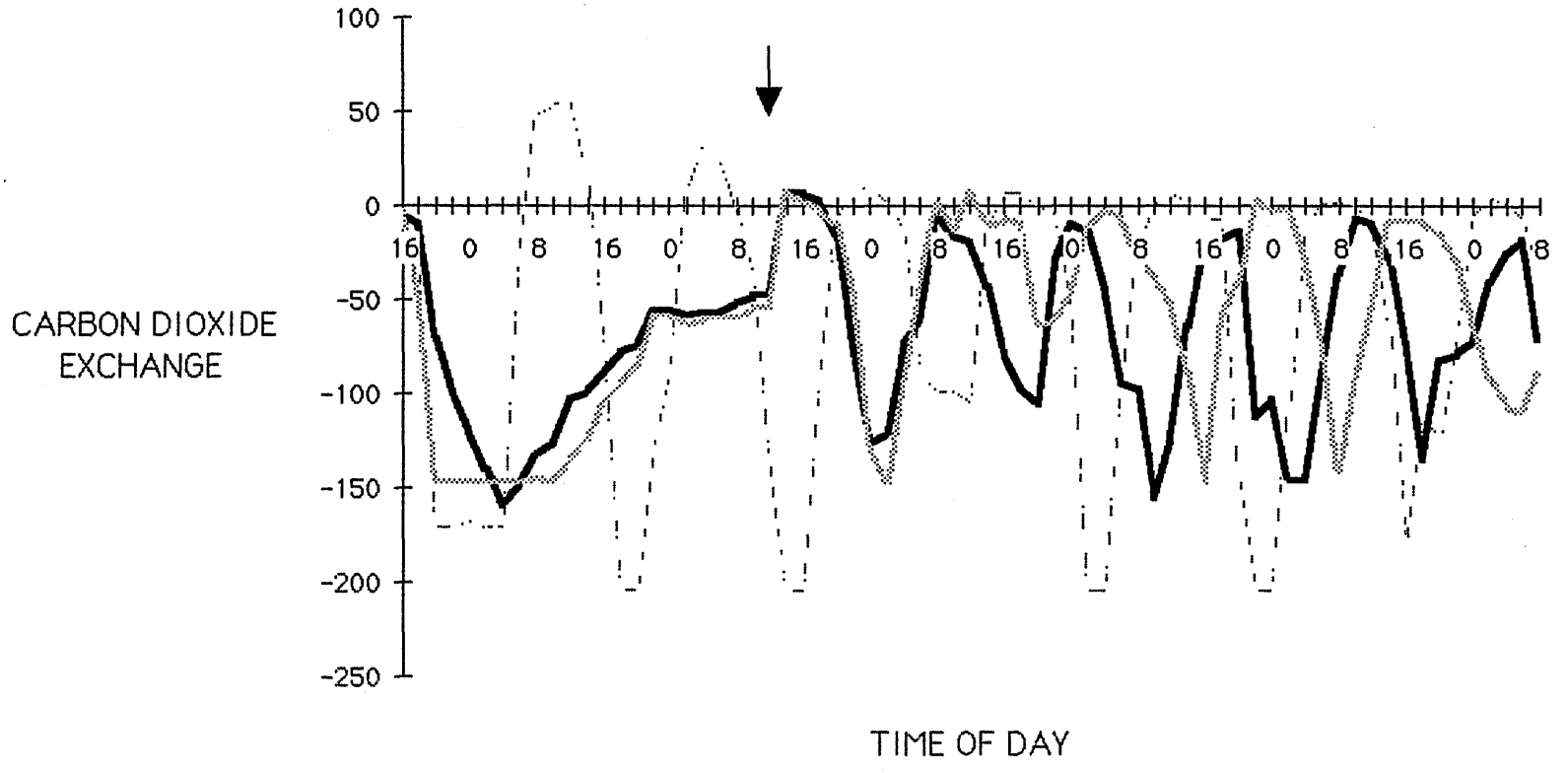

CARBON DIOXIDE EXCHANGE
100
50
0
-50
-100
-150
-200
-250
16
0
8
TIME OF DAY

FIGURE 3.18. Inhibition of the rhythm of CO_2 exchange in two samples of leaves of *Bryophyllum fedtschenkoi* held simultaneously at 2°C and its restoration on increasing the temperature to 15°C. The broken line represents the rhythm in control leaves held continuously at 15°C. Conditions and presentation are as described for Fig. 3.17 except that the temperature change from 2 to 15°C was made at midnight.

FIGURE 3.18

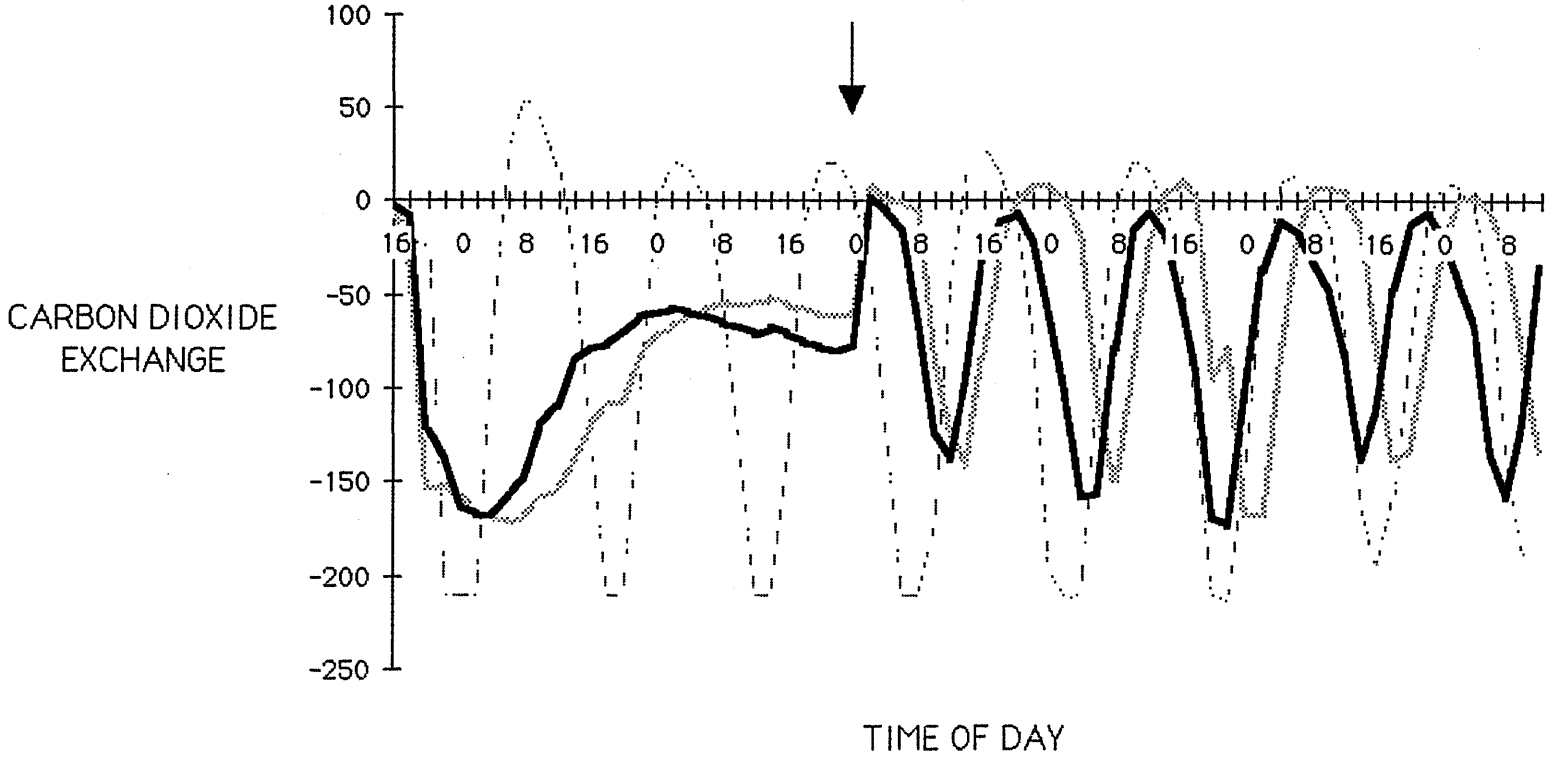
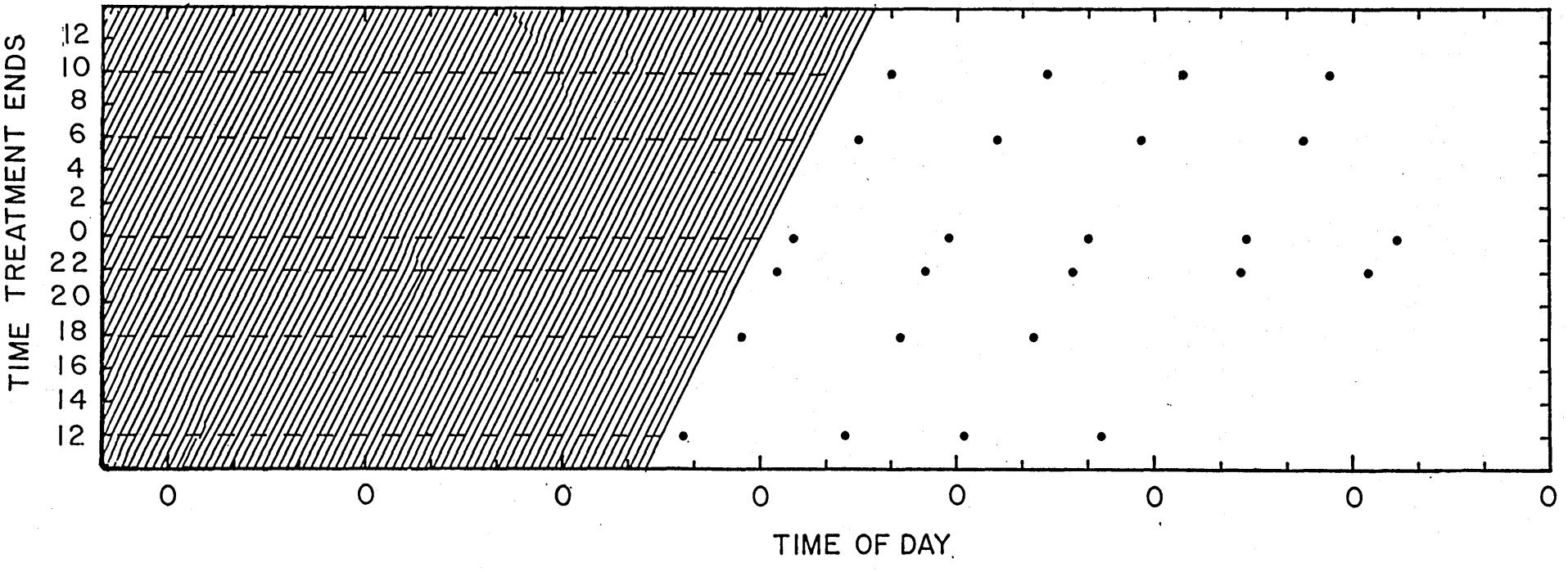

CARBON DIOXIDE EXCHANGE
100
50
0
-50
-100
-150
-200
-250
16
0
8
TIME OF DAY

FIGURE 3.19. Collated data for a series of experiments in which leaves were held at 2°C for a few days then transferred to 15°C at various times as indicated by the end of the shading. Points on any horizontal line show the mean times of occurrence of peaks in at least two samples of leaves following a treatment at the time indicated on the ordinate , 0 = midnight.


TIME TREATMENT ENDS
12
10
8
6
4
2
0
22
20
18
16
14
12
0
0
0
0
0
0
0
0
TIME OF DAY

Moreover, the results indicate that at 2°C inhibition is achieved by the oscillator being held at a fixed phase point from which oscillation always begins when the temperature is increased to 15°C.

The period of the newly initiated rhythm in leaves at 15°C after exposure for up to two days to 2°C was 17.8 ± 0.25 h, a value significantly shorter than the period of 18.9 ± 0.15 h recorded in leaves which were maintained at 15°C throughout the experiment. This finding suggests that the oscillator may speed up after prolonged exposure to low temperature.

The most important finding to emerge from this section of the investigation was that following exposure to a high temperature treatment the rhythm always began with an initial burst of CO_2 uptake to give a trough in the rhythm, whilst the rhythm initiated after exposure to a low temperature treatment began with a decrease in the rate of CO_2 uptake to provide a peak in the rhythm. As a result, for leaves transferred to 15°C at a particular time, the phases of the rhythms of CO_2 exchange in leaves previously held at 40 and 2°C are reversed. The basic oscillating mechanism must therefore be driven to, and held at, fixed phase points by these temperatures, and furthermore, the fixed phase points attained at 40°C and 2°C must differ by a phase angle of 180°.

The next stage in this investigation was to identify the fixed phase points to which the oscillator is driven by 40 and 2°C in terms of the malate hypothesis outlined in the introduction. The results presented above indicate that these fixed phase points may be associated with, or characterised by the malate status of the leaf cells. At 40°C, no CO_2 fixation occurs and the leaf cells are therefore likely to have a low malate status, bearing in mind that they were exposed to this temperature at the end of the photoperiod when their malate status would already be low. This low malate status would be consistent with the immediate onset of CO_2

fixation when the leaves are transferred to 15°C. At 2°C on the other hand, a large amount of CO_2 fixation takes place, and the malate status of the leaf cells might be expected to be high, despite there being a low level of malate in the cells when the experiment began. The fact that the rhythm begins on transfer to 15°C with a large decrease in the rate of CO_2 uptake is also consistent with the high malate status of the leaf at 2°C, the decarboxylation of this malate being required before further CO_2 fixation by PEPCase can take place.

In order to establish whether or not these deductions were correct the malate status of the leaves was determined by procedure No.1 outlined in the Methods section of this thesis.

The concentration of malate in the sap from leaves held at 40°C and 2°C has been determined as a function of time, and these results are shown in Fig. 3.20.

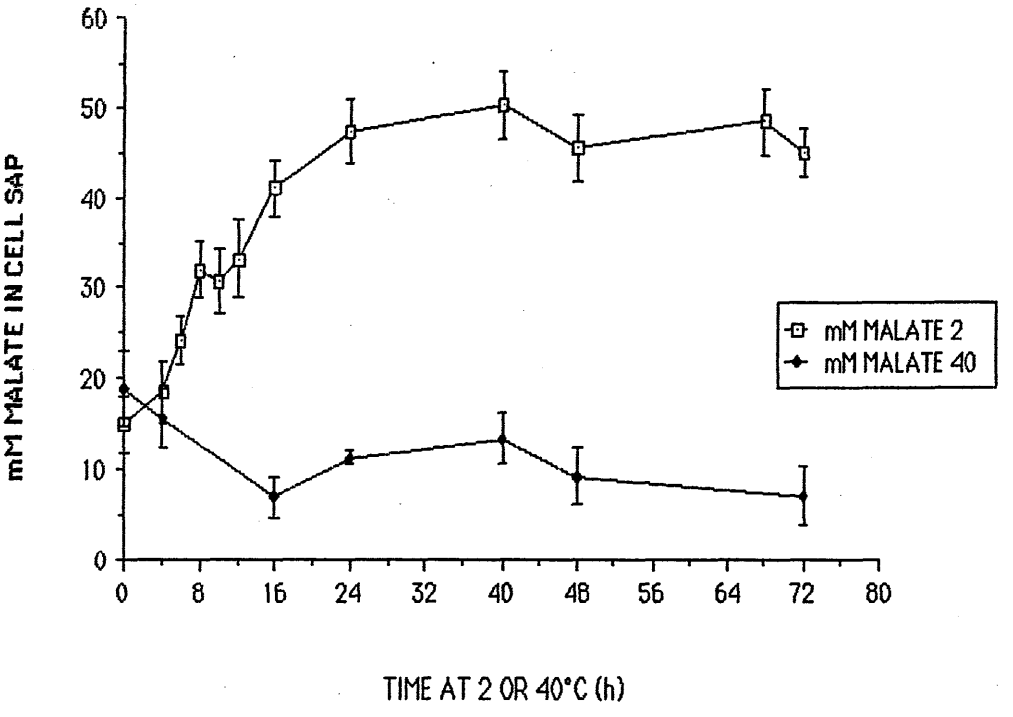


FIGURE 3.20. Concentration of malate in the extracted cell sap of leaves of *Bryophyllum fedtschenkoi* maintained in a stream of normal air, continuous illumination and at either 40 or 2°C as indicated in the box. The vertical lines indicate ± the standard error of the mean values obtained from at least 6 leaves. Ordinate: concentration of malate in mM. Abscissa: No. of hours at 2 or 40°C.

In order to compare the change in malate content of the leaves with their rate of CO_2 fixation as a function of time the results have been replotted overlaying the CO_2 exchange curves recorded during these experiments in Figs. 3.21 A and 3.21 B.

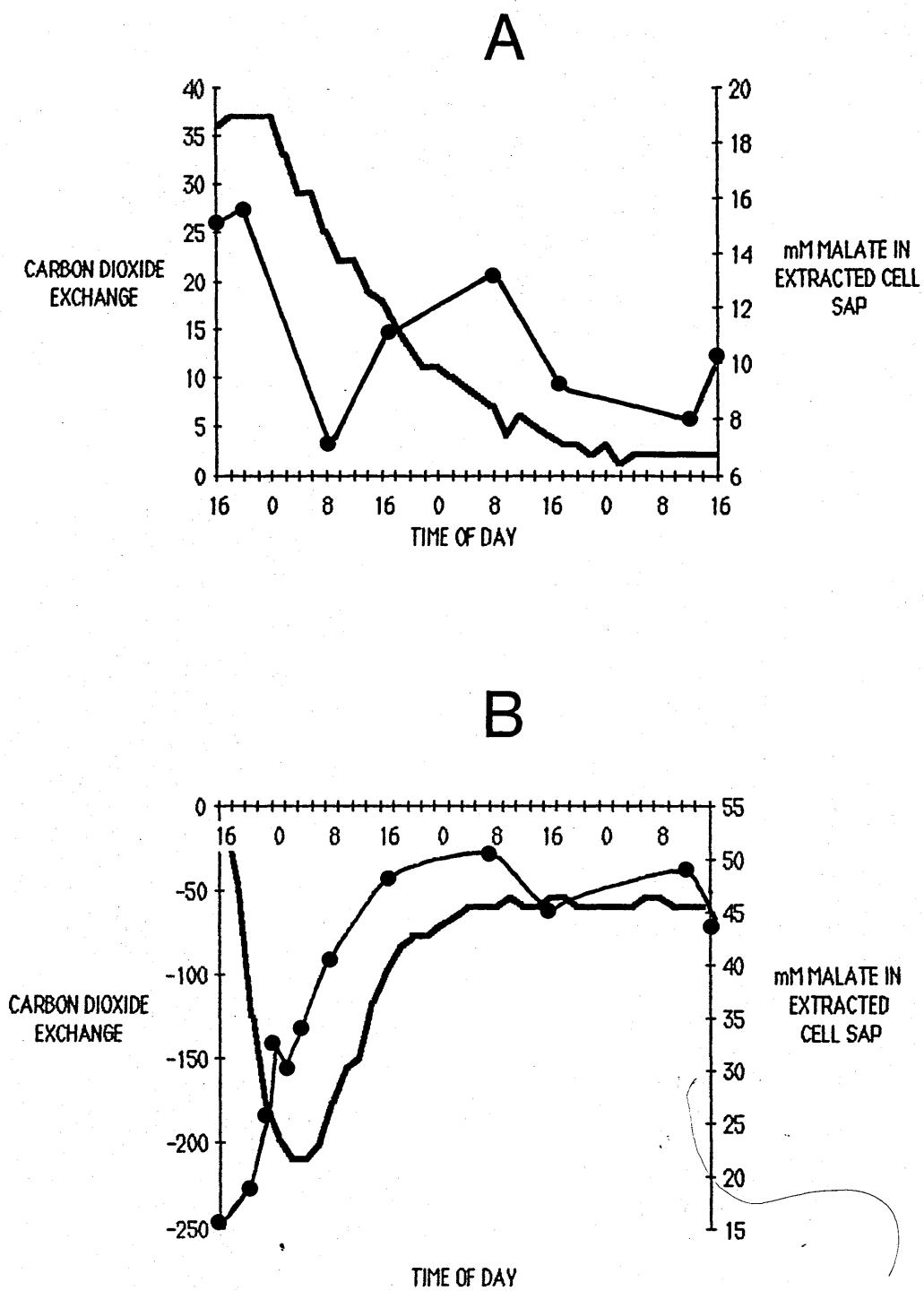


FIGURE 3.21A & B. The malate status of leaves maintained in light and normal air at 40°C (A) or 2°C (B) (●) and the pattern of CO_2 assimilation exhibited by leaves kept under those conditions (continuous line). Ordinate (left): the rate of uptake of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}$ g (fresh weight)$^{-1}$ (right) concentration of malate (mM) in the extracted cell sap. Abscissa : time of day 0 = midnight.

The malate status of leaves held at 40°C for 72 h remains relatively low as determined from the malate concentration of the expressed sap which varied between a maximum value of 19 mM and a minimum value of 7 mM. Significant differences were not found between any of the points on this curve.

For leaves held at 2°C, the concentration of malate in the extracted cell sap increased markedly from approximately 15 mM at the start of the experiment to a maximum level of 45 - 50 mM 24 h later and remained constant thereafter. This high, uniform concentration of malate is highly significantly different from the lower level found in the sap of leaves held at 40°C.

The kinetics of the changes in CO_2 assimilation and malate synthesis are compared in Figs. 3.21B and B. It should be noted that the rate of CO_2 uptake at 2°C increases to a maximum value at 0400 h on the second day of the experiment, which corresponds to the time at which the malate content of the cell sap has reached half its maximum value. From 0400 h onwards, the rate of CO_2 fixation declines and the rate of malate accumulation also begins to decline shortly afterwards. CO_2 fixation reaches a constant rate at approximately the same time at which the concentration of malate reaches a constant maximum value, that is about 24 h after the beginning of the experiment. At 40°C, the CO_2 output of the leaves gradually declines while their malate status remains at a low constant value.

Measurements were also made of the pH of the cell sap which was used to determine the malate concentrations at 2°C. The results are shown in Fig. 3.22.

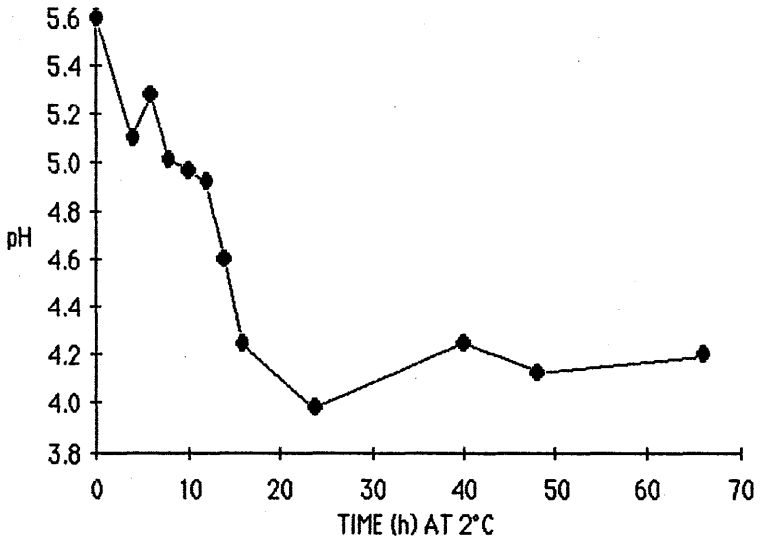


FIGURE 3.22. pH of the extracted cell sap of leaves maintained in a stream of normal air and continuous illumination at 2°C. Each point represents the mean of at least 6 leaves. Ordinate: pH. Abscissa: No. hours at 2°C.

The pH of the cell sap decreased from 5.6 to 4.0 over the first 24 h at 2°C, and thereafter remained relatively constant. Thus, the kinetics of the changes in the pH of the cell sap are indistinguishable from the kinetics of its malate content.

These data have therefore enabled the two fixed phase points at which the oscillator is held at 40°C and 2°C to be characterised in terms of the malate status of the leaf. The findings are entirely consistent with the malate states deduced from the CO_2 exchange data presented in Figs. 3.15, 3.16, 3.17 and 3.18, and the phases of the rhythms which begin when the leaves are transferred to 15°C. At 40°C the malate status of the leaf is low, and at 2°C it is high.

The accuracy of the procedure employed to extract the cell sap from the leaves in the above experiments depends, however, upon the acidity of the leaves being sufficiently high to inhibit enzyme activity. Since the levels of malate recorded in

leaves held at 40°C were relatively low, it was appreciated that enzyme activity may not have been inhibited on extraction thus giving rise to false readings. This possibility was tested by comparing malate levels in leaves extracted by the two procedures outlined in the Methods section, and referred to here as the "garlic press" method and the "perchloric acid" method. The presence of perchloric acid in the extraction mixture ensures that enzyme activity is inhibited.

Six leaves were subjected to 40°C for 24 h; they were then removed from the leaf chambers and cut in half along the midrib. One half was extracted using the "garlic press" method and the other half using the "perchloric acid" method. Half-leaves were used to minimise the variability resulting from the malate content in different leaves. The malate content of the half-leaves extracted by the two methods was then compared. The results of two independent experiments (1st two rows) and the average results of both (3rd row) are shown in Table 4.

TABLE 4

COMPARISON OF MALATE LEVELS IN LEAVES PREPARED BY TWO DIFERRENT EXTRACTION PROCEDURES.

CONCENTRATION OF MALATE IN CELL SAP(mM)			
GARLIC PRESS METHOD	PERCHLORIC ACID METHOD	t	S
6.7 ± 0.92	6.4 ± 1.15	0.235	N.S.
10.7 ± 1.64	12.0 ± 1.75	0.527	N.S.
8.9 ± 1.13	9.2 ± 1.31	0.160	N.S.

These results clearly indicate that there is no significant difference in the levels of malate obtained using the two methods for extracting the leaves and

confirm Nimmo's finding (Nimmo, unpublished data). As the garlic press method was simpler and less time consuming this procedure was used to prepare leaves for all subsequent malate assays.

The results presented above indicate that high temperature inhibits the rhythm of CO_2 exchange by holding the basic oscillator in a state in which the leaves contain relatively little malate. In contrast, low temperature appears to prevent the operation of the rhythm by holding the oscillator in a state in which the leaves contain relatively high levels of malate. These findings suggest that malate synthesis is inhibited by high temperature whilst malate removal is inhibited by low temperature.

A further point of interest was to establish whether high temperature, in addition to inhibiting malate synthesis, also prevented malate breakdown. No information on this point was provided by previous experiments since leaves were always placed in 40°C at the end of the photoperiod when their malate status was already low. A number of experiments were therefore conducted in which the malate status of leaves held at 2°C for 44 h was compared with that of leaves in which the 2°C treatment was followed by a 40°C treatment of various durations. If the enzyme responsible for malate breakdown is active at 40°C then a decrease in malate levels following exposure to high temperatures would be expected. The results of this study are shown in Fig. 3.23.

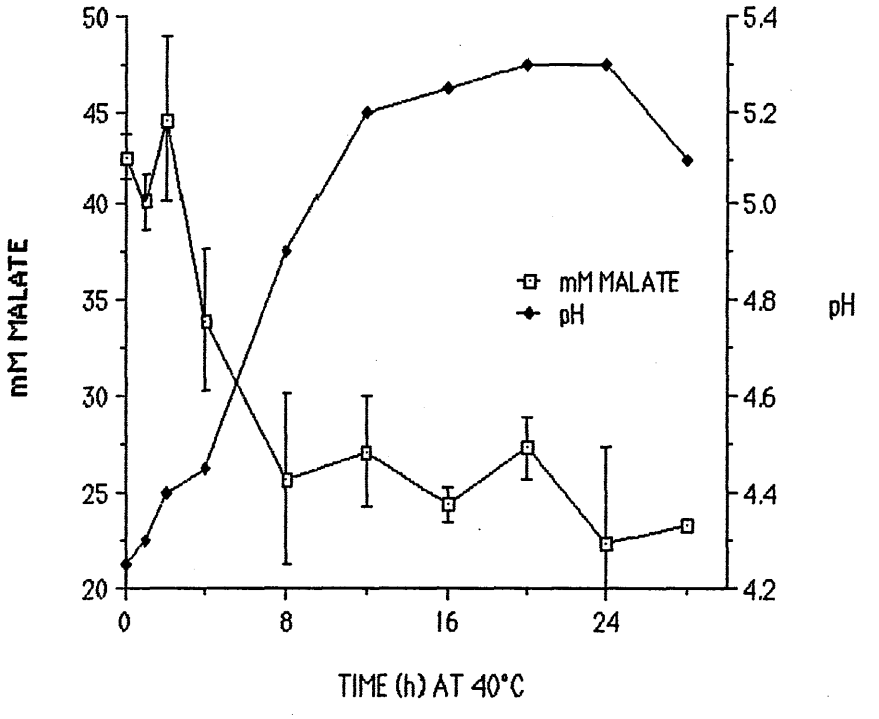


FIGURE 3.23. Disappearance of malate from leaves exposed to 40°C for various lengths of time following a 44-h exposure to 2°C, and the corresponding changes in the pH of the sap. Ordinate: concentration of malate in extracted cell sap in mM (left) and pH (right). Abscissa: time at 40°C in hours.

These results indicate that little or no change in the malate content of the leaves occurs within the first 2 h of the change in temperature from 2 to 40°C. After this time, however, the malate concentration of the cell sap begins to decline and reaches a minimum value of approximately 25 mM 8 h after the temperature change. Extending the 40°C treatment beyond 8 h does not appear to decrease the malate content of the leaves further, the sap concentration remains at 20 - 25 mM. The decrease in malate content was reflected more or less in the pH of the cell sap which increased from approximately 4.2 just prior to transferring leaves from 2 to 40°C to 5.2 12 h later, 4 h after the level of malate had become constant. Thereafter the pH remained more or less constant.

These results establish that the enzyme system responsible for malate breakdown is indeed active at 40°C. Within 8 h of transferring leaves from 2 to 40°C the concentration of malate in the extracted sap decreases from approximately 45 mM to 25 mM, a concentration consistent with that occurring in leaves extracted from plants in the growth cabinet at the end of the normal photoperiod.

Previous results have shown that the phases of rhythms which begin on transferring leaves at particular times from either 40 or 2°C to 15°C are 180° different. It follows therefore, from the malate data presented above, that a rhythm in a leaf which is subjected first to 2°C for a few days, then to 40°C for 8 h before being transferred to 15°C should be 180° out of phase with a rhythm in a leaf transferred directly from 2 to 15°C at the same time, because the 8-h, 40°C treatment would reduce the malate level in leaves to a low value. On the other hand, a rhythm in a leaf commencing after transfer to 15°C following exposure to 2°C for 44 h and then 40°C for only 4 h should not be 180° out of phase with a rhythm in a leaf transferred from 2 to 15°C directly because the 4-h 40°C treatment is long enough to reduce only partially the malate content. It would, however, be anticipated that the first peak of the rhythm of CO_2 exchange initiated in a leaf transferred to 15°C following sequential exposure to 2°C for 44 h and 40°C for 4 h would occur later than that in leaves transferred directly from 40 to 15°C because the leaves would have to metabolise the remaining malate before CO_2 fixation could begin. A number of experiments were therefore carried out in which leaves were exposed first to 2°C for 44 h and then to 40°C for either 4 or 8 h. The phase of the rhythm initiated in these leaves after transferring them to 15°C was compared with the phase of the rhythm initiated in leaves transferred directly from 2 to 15°C at the same time.

Representative results of these experiments are shown in Figs. 3.24 and 3.25. The dark line represents the rhythm in leaves subjected to either 40°C for 8 h (Fig. 3.24) or for 4 h (Fig. 3.25) before being transferred to 15°C and the shaded line that in leaves transferred directly from 2 to 15°C.

FIGURE 3.24. Inhibition of the rhythm of CO_2 exchange in leaves held at $2^{o}C$ and its restoration on increasing the temperature to $15^{o}C$ (shaded line). The dark line represents the restoration of the rhythm at $15^{o}C$ in leaves in which the $2^{o}C$ treatment was followed by an immediate exposure to $40^{o}C$ for 8 h. The first arrow indicates the time at which the temperature change from 2 to $40^{o}C$ was made (midday) and the second arrow the time at which both samples of leaves were placed in $15^{o}C$ (2000 h). Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.24

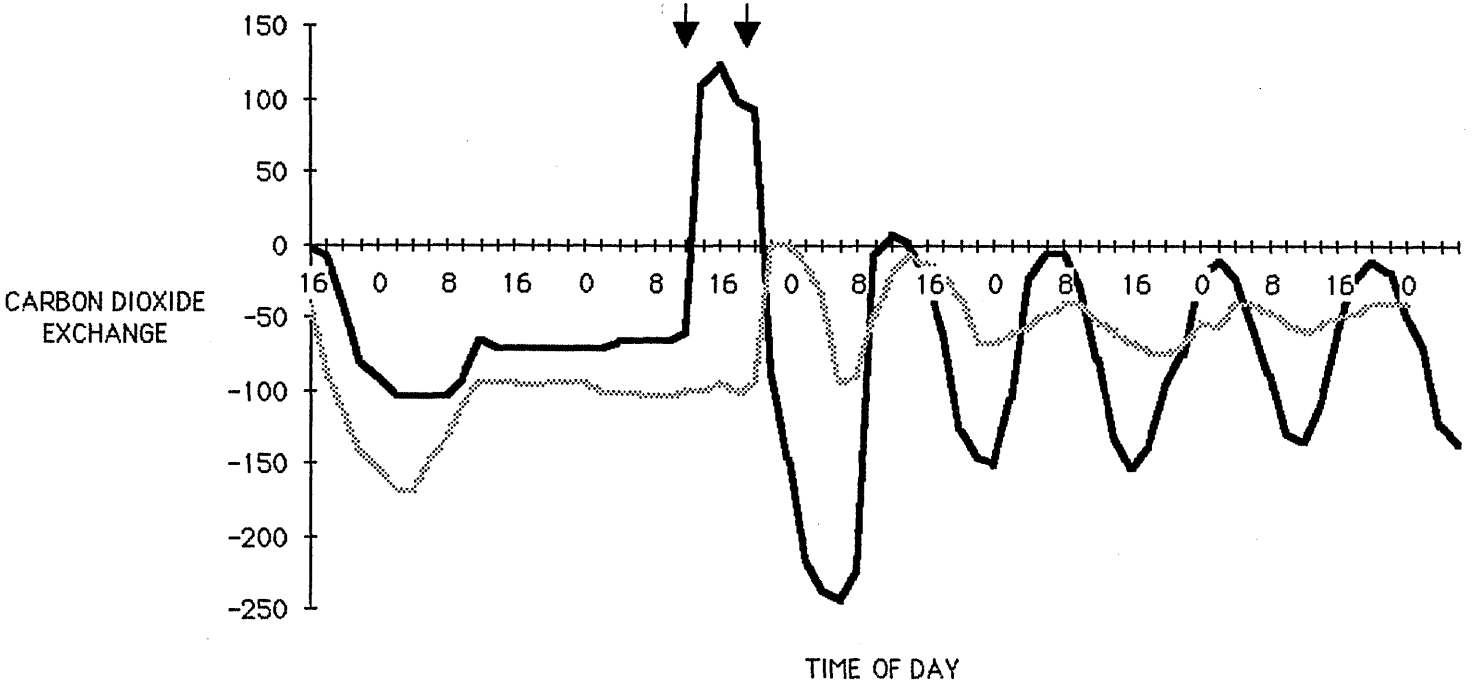
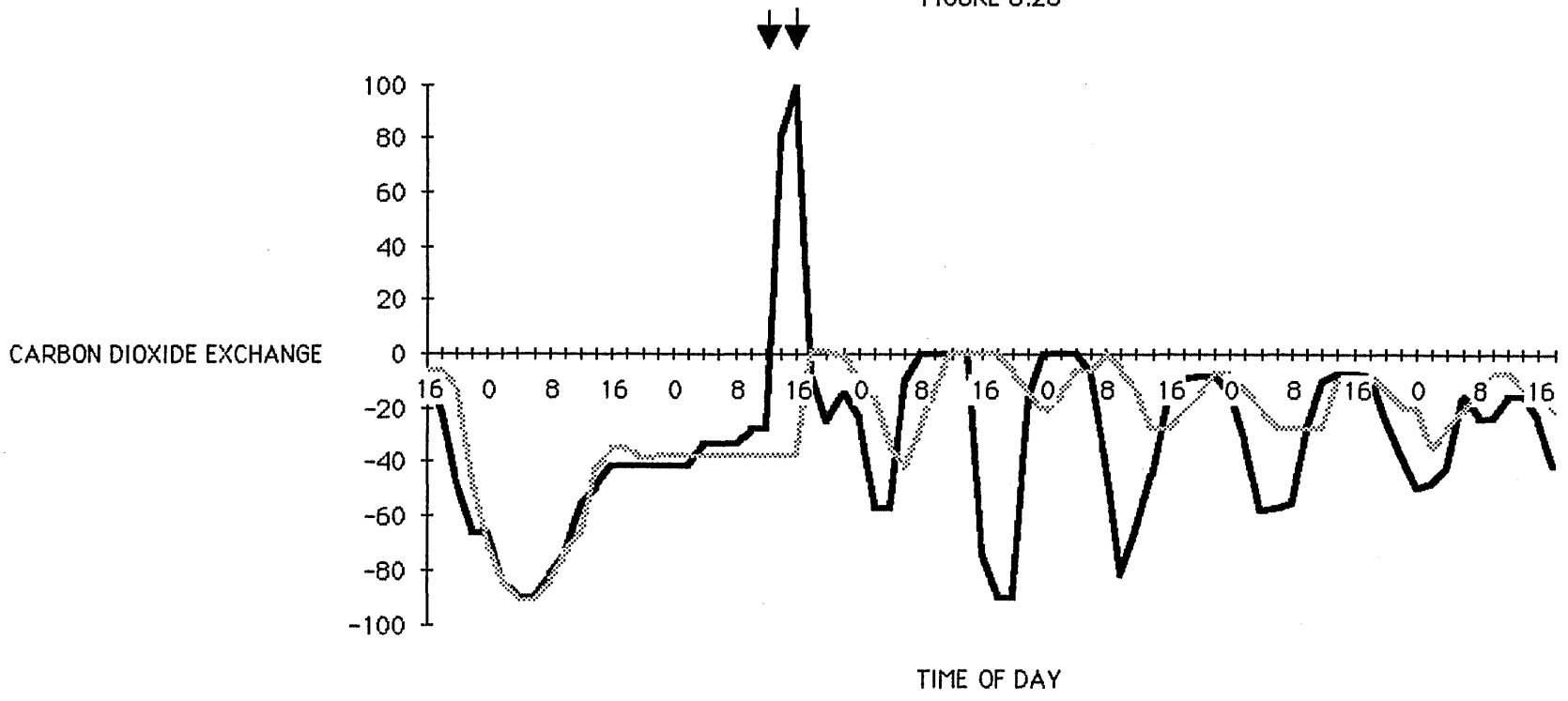

150
100
50
0
-50
-100
-150
-200
-250
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.25. Inhibition of the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* held at 2°C and its restoration on increasing the temperature to 15°C (shaded line). The dark line represents the restoration of the rhythm at 15°C in leaves in which the 2°C treatment was followed by an immediate exposure to 40°C for 4 h. The first arrow indicates the time at which the temperature change from 2 to 40°C was made (midday) and the second arrow the time at which both samples of leaves were placed in 15°C (1600 h). Leaves were otherwise maintained in normal air and continuous light. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in μg CO_2 h^{-1} g (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.25


CARBON DIOXIDE EXCHANGE
100
80
60
40
20
0
-20
-40
-60
-80
-100
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
TIME OF DAY

The results in Fig. 3.24 indicate that the rhythms initiated in leaves on transfer to 15°C do indeed begin out of phase although the difference in phase is less than 180°. The first peak of the rhythm in leaves exposed to 40°C occurred approximately 17 h after the transfer to 15°C, 2 - 3 h later than the trough of the rhythm in leaves transferred from 2 to 15°C directly. The phase angle difference between the rhythms in the two samples of leaves does not remain constant due to a difference in the lengths of the periods. The period of the rhythm in the 40°C treated leaves, 19.4 ± 0.50 h, is significantly longer than the period of 18.1 ± 0.20 h recorded in leaves transferred directly from 2 to 15°C.

In Fig. 3.25, when leaves were exposed to 2°C for 44 h then to 40°C for 4 h before being transferred to 15°C, the first peak of the rhythm occurred approximately 18 h after the transfer, 3 h later than the average time of occurrence of the first peak in leaves transferred directly from 40 to 15°C (see Table 3). In leaves transferred directly from 2 to 15°C, the first peak occurred approximately 4 h after the temperature change. As anticipated the rhythms were not 180° out of phase. The difference in period length between the two samples of leaves accounts for the fact that the phase angle difference between the two rhythms does not remain constant during the time at which the leaves were maintained at 15°C. The length of the period in the leaves exposed to 40°C was 17.9 ± 0.30 h, which was significantly longer than the period of 16.3 ± 0.39 h recorded in leaves transferred directly from 2 to 15°C. The latter period is, however, somewhat shorter than that of 17.8 ± 0.25 h recorded previously (See page 75), and since it was determined from only two experiments its validity is questionable.

Thus, the results presented in Figs. 3.23 - 3.25 indicate that the enzyme system responsible for malate breakdown is capable of operating even when leaves are subjected to a temperature of 40°C. Exposing malate-rich leaves to 40°C for 8 h reduces the malate level to a value consistent with the upper range of that occurring in leaves extracted at the end of the normal photoperiod i.e. about 20 -

25 mM.

The time which elapsed between transferring leaves directly from 40 to 15°C (Table 3) and the occurrence of the first peak was approximately 15 h. The data presented above show that the first peak in leaves exposed to 2°C for 44 h and then 40°C for 8 h occurs approximately 17 h after the leaves were transferred to 15°C whilst in leaves exposed first to 2°C then to 40°C for 4 h the first peak occurs approximately 18 h after the end of the high temperature treatment. It appears therefore that the longer the 40°C treatment, the less malate is present in the leaf cells and the less time required to reach the first peak on transferring leaves to 15°C.

3.1.2.2. The Effects of Short Pulse-Type Temperature Treatments

In this section an attempt has been made to identify the phase points in the cycle at which the oscillator is sensitive to high and low temperatures by subjecting leaves maintained at 15°C to either 40 or 2°C for 4 h at various points in the cycle.

The continuous lines in Figs. 3.26 - 3.29 represent the results obtained when the temperature was increased from 15 to 40°C for 4 h at various points in the cycle. The broken line indicates the rhythm in control leaves held continuously at 15°C. In Figs. 3.27 and 3.29 the magnitude of phase shift on successive peaks appears to decrease and increase respectively. This effect is due to a slight increase in the period observed after the short exposure to high temperature and will be discussed more fully later.

In Fig. 3.26 the temperature was increased for 4 h from 0800 h to midday across a peak in the rhythm of CO_2 exchange. Exposing leaves to high temperature in this position in the cycle clearly has no effect on the phase of the rhythm. The first post-treatment peak occurred approximately 18 h after the end of the 40°C

FIGURE 3.26. The effect of exposing two samples of leaves of *Bryophyllum fedtschenkoi* to 40°C for 4 h, from 0800 h to midday, as indicated by the arrows. Leaves were otherwise maintained in continuous illumination and a stream of normal air at 15°C. The rhythms in two samples of treated leaves are represented by the continuous lines and those in untreated control leaves by the broken line. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.26

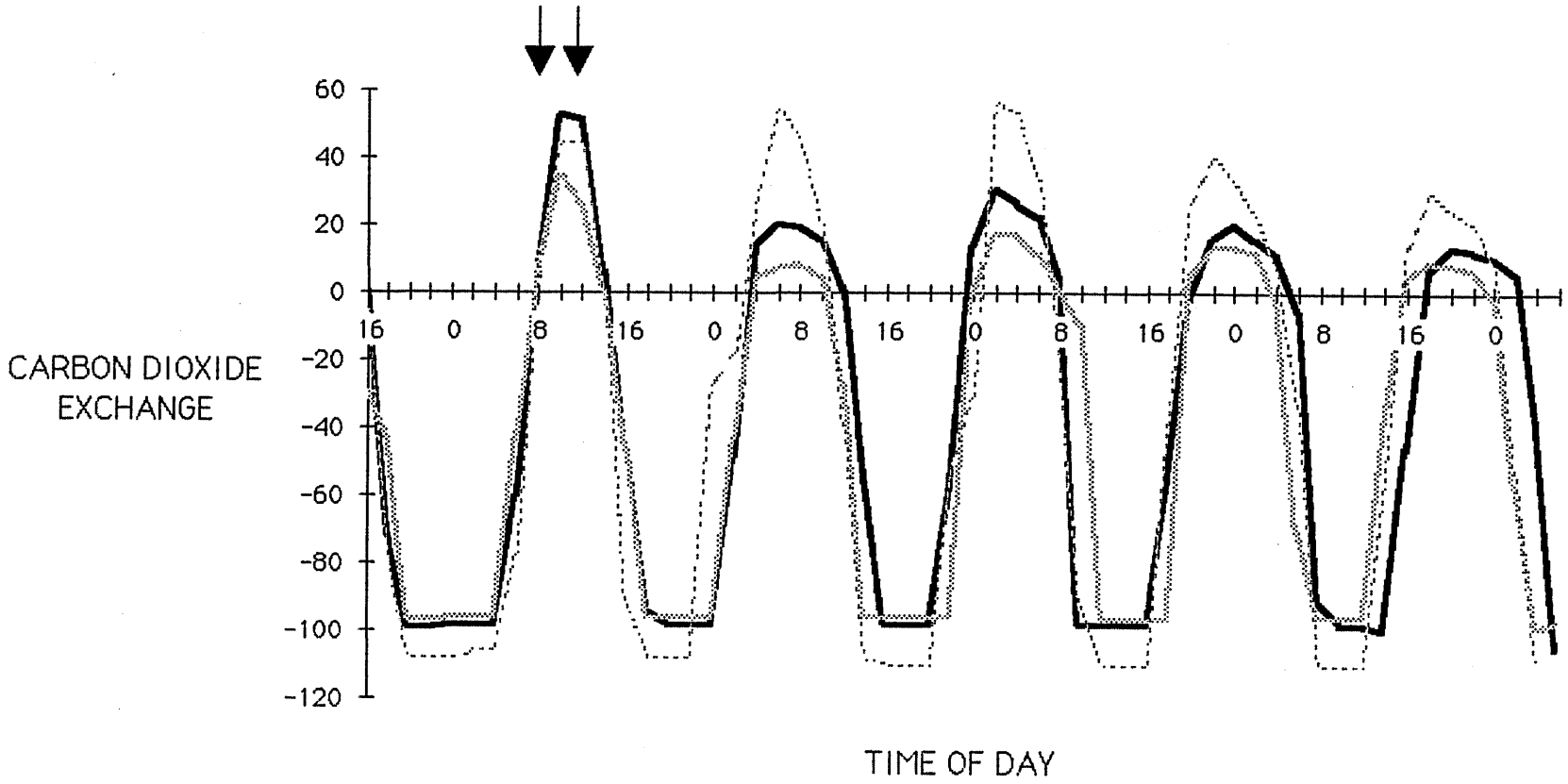

CARBON DIOXIDE EXCHANGE
60
40
20
0
-20
-40
-60
-80
-100
-120
16
0
8
16
0
8
16
0
8
16
0
8
16
0
TIME OF DAY

FIGURE 3.27. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination and a stream of normal air at 15°C by exposure to 40°C for 4 h from 2000 h to midnight as indicated by the arrows (continuous lines). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.26.

FIGURE 3.27

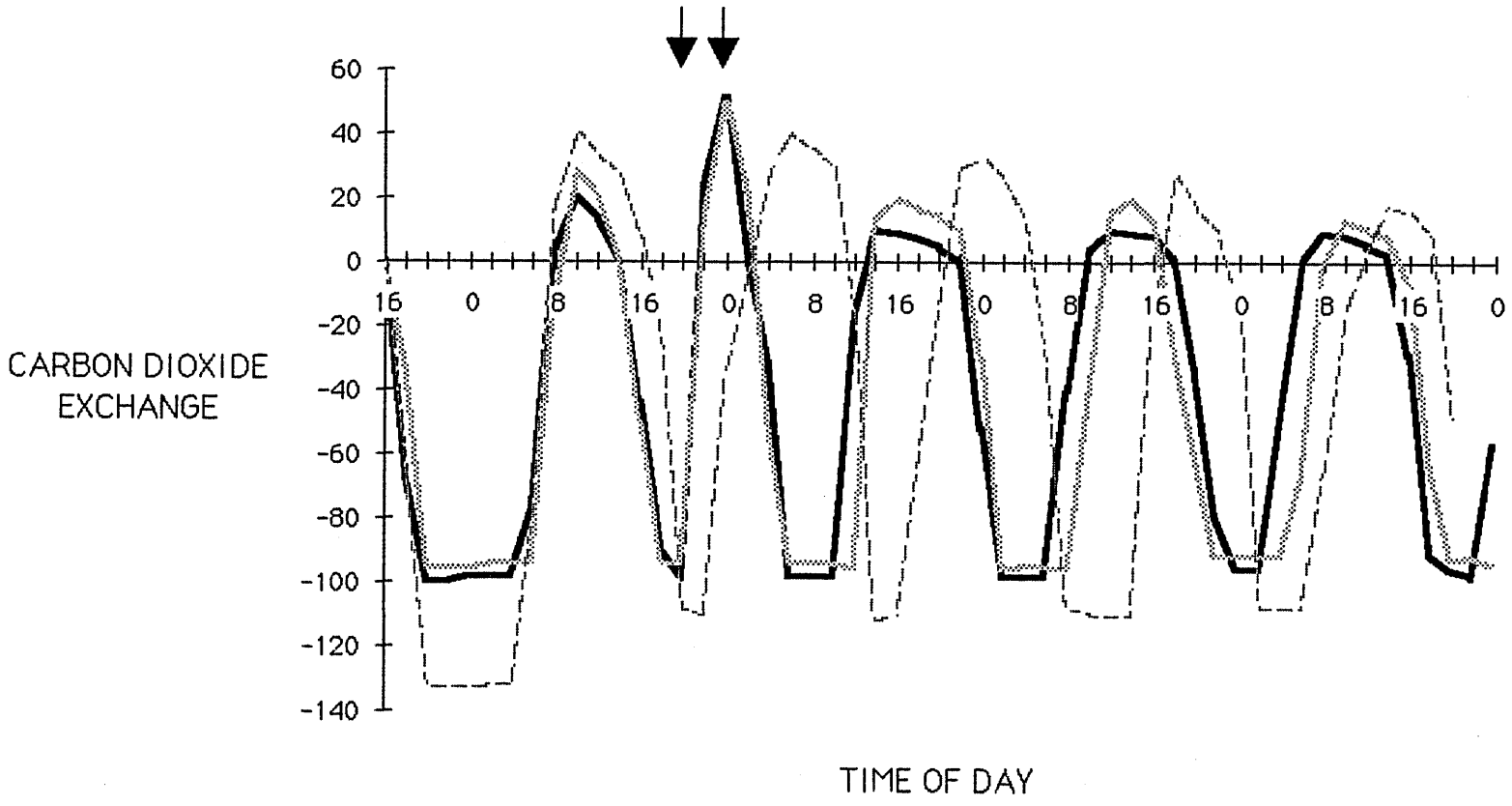

CARBON DIOXIDE EXCHANGE
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
16
0
8
16
0
8
16
0
8
16
0
8
16
0
TIME OF DAY

FIGURE 3.28. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination and a stream of normal air at 15°C by exposure to 40°C from midnight to 0400 h as indicated by the arrows (continuous lines). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.26.

FIGURE 3.28

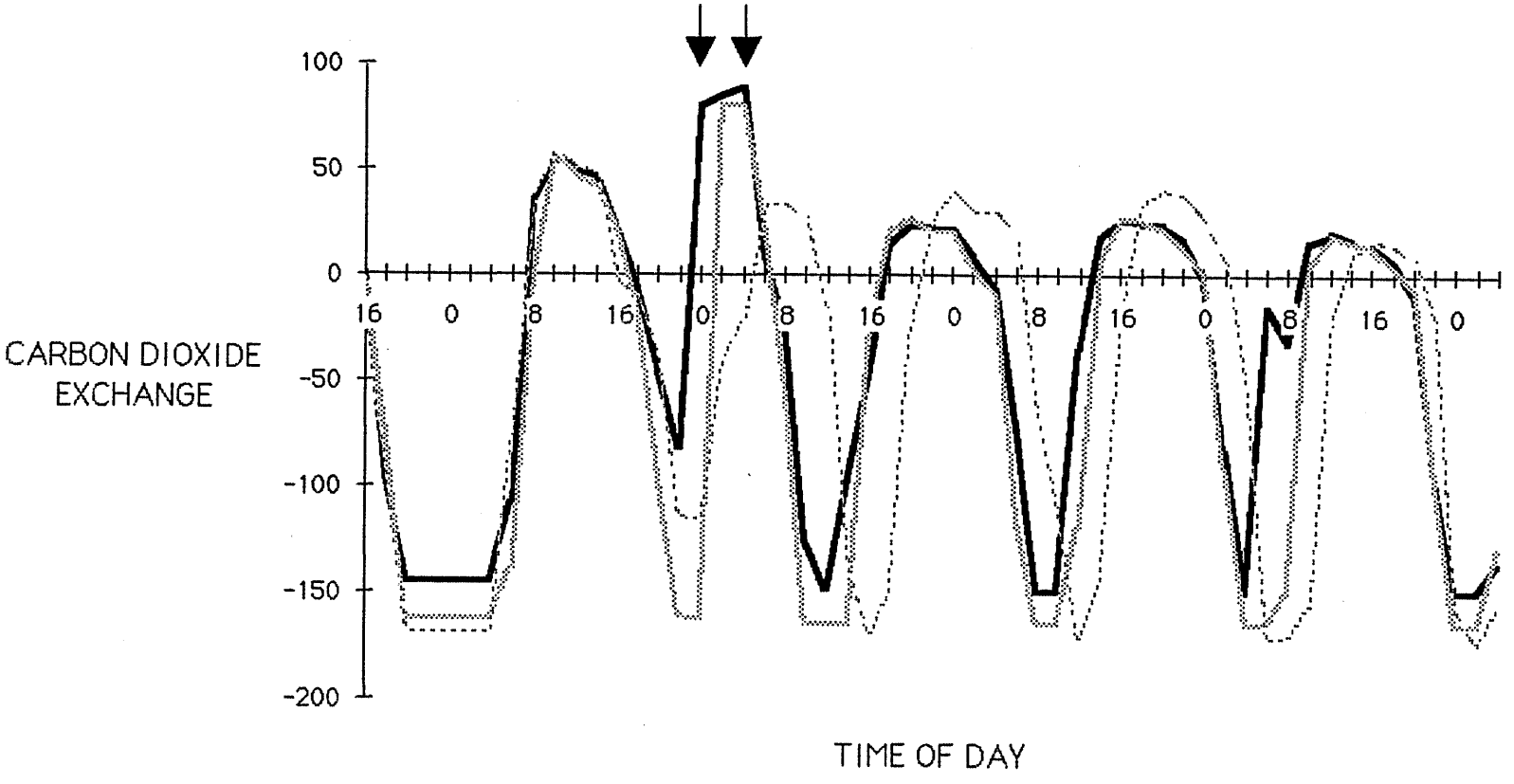
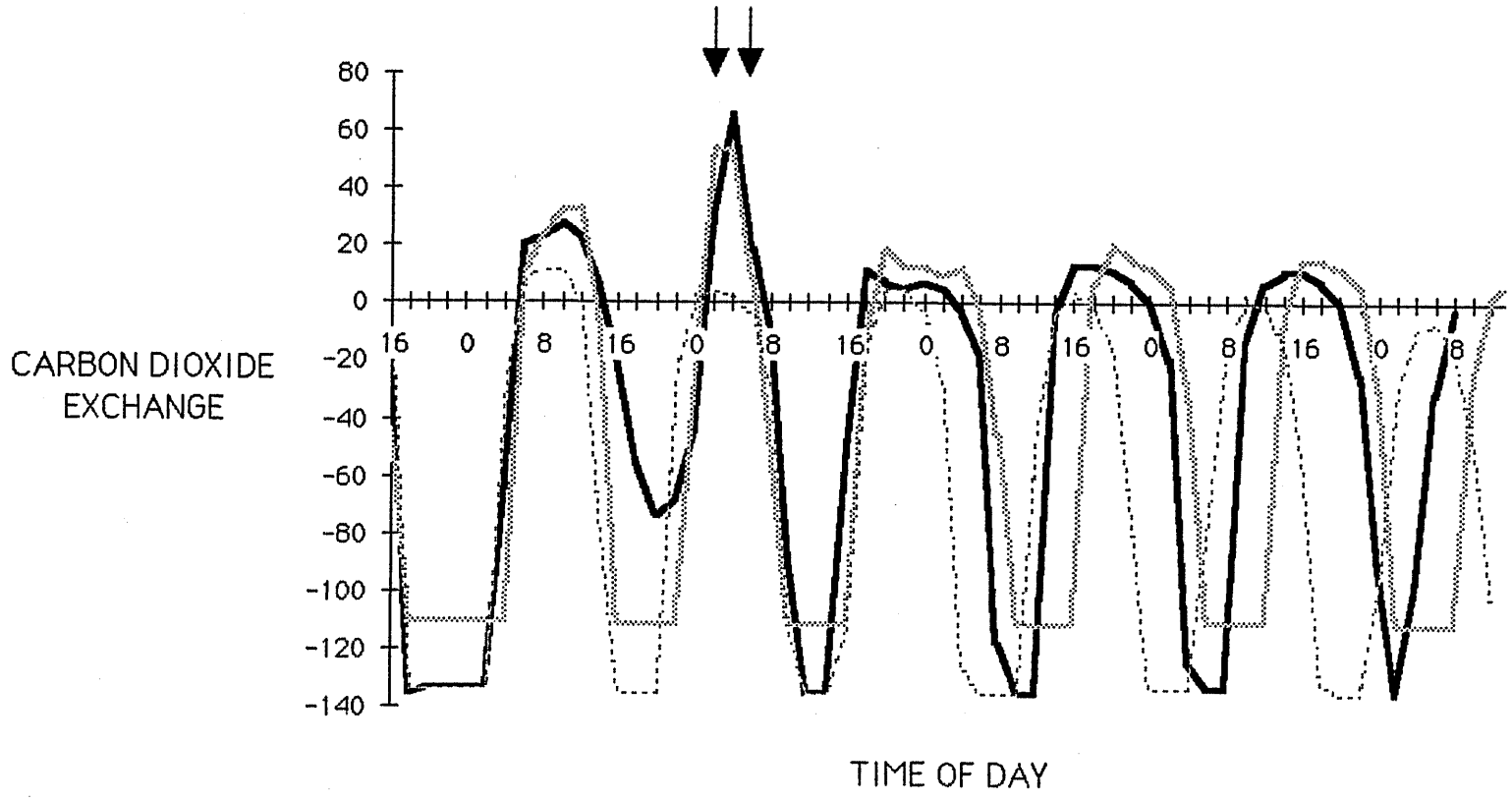

100
50
0
-50
-100
-150
-200
16
0
8
16
0
8
16
0
8
16
0
8
16
0
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.29. Phase shifts induced in the rhythm of CO_2 exchange in two samples of leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination and a stream of normal air at 15°C by exposure to 40°C from 0200 h to 0600 h as indicated by the arrows (continuous lines). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.26.

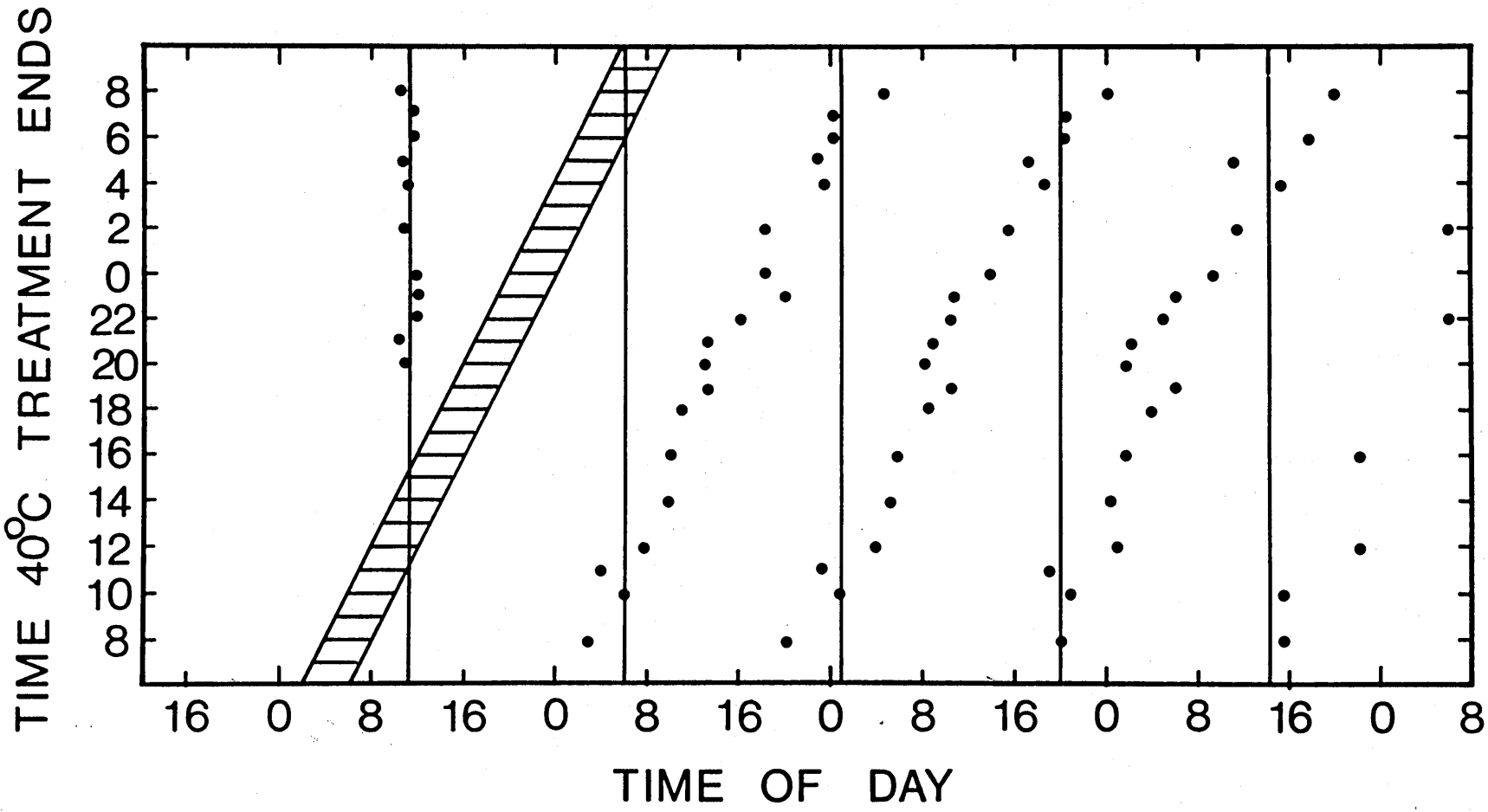
FIGURE 3.29


CARBON DIOXIDE EXCHANGE
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
TIME OF DAY

treatment and coincided with the peak in the control leaves; subsequent peaks occurred at intervals of approximately 19.0 h. In contrast, when leaves were exposed to 40°C between 2000 h and midnight as shown in Fig. 3.27, a large phase shift occurred. During the 40°C treatment the rate of CO_2 uptake decreased, CO_2 uptake being replaced by CO_2 output 1-2 h after the temperature increase took place. On restoring the temperature to 15°C, there was an immediate increase in the rate of CO_2 uptake and the first peak of the rhythm occurred approximately 17 h after the end of the treatment. A phase shift was also induced when leaves were exposed to 40°C from midnight to 0400 h. The results of this experiment are shown in Fig. 3.28. The CO_2 exchange pattern during and after the high temperature treatment is essentially similar to that described for Fig. 3.27 and again the first peak occurred approximately 17 h after the end of the high temperature treatment. When the high temperature treatment ended at 0600 h, as shown in Fig. 3.29 a small phase shift also occurred. The gradual difference in the times of occurrence of successive peaks in the two treated leaf samples in this experiment is due to the period in one sample being slightly longer than the period in the other.

The collated data for a number of experiments in which the whole cycle was scanned at intervals of at least two hours with treatments in which the temperature was increased from 15 to 40°C for 4 h are presented in Fig 3.30. These data show that the magnitude of the phase shift induced by a 4-h, 40°C treatment is dependent upon the position in the cycle at which the treatment ends. The first peak following a high temperature treatment always occurs approximately 17 h after the end of the treatment, regardless of the time of day at which the treatment is administered. Maximum phase shifts are induced by treatments ending between the peaks of the rhythm, while little or no phase shift is induced by treatments ending across a peak in the rhythm. These results support the view that high temperature shifts the phase of the rhythm by forcing the basic oscillator to, and holding it at, a

FIGURE 3.30. Collated data for a series of experiments in which the cycle of CO_2 exchange in *Bryophyllum* leaves was scanned at approximately hourly intervals with a 4-h treatment during which time the temperature was raised to 40°C. Leaves were otherwise maintained in continuous light and a stream of normal air at 15°C. Times of occurrence of the peaks of untreated control rhythms are represented by the vertical lines. Points in any horizontal line show the mean times of occurrence of the peaks of the rhythms in at least two samples of leaves following a treatment at the time indicated on the ordinate. Treatment times are indicated by the shaded bar.


TIME 40°C TREATMENT ENDS
8
6
4
2
0
22
20
18
16
14
12
10
8
16 0 8 16 0 8 16 0 8 16 0 8 16 0 8
TIME OF DAY

fixed phase point in the cycle corresponding to a peak in the rhythm of CO_2 exchange and are consistent with the results reported for prolonged exposures to high temperature. It should be noted, however, that the time taken to reach the first peak at 15°C, following prolonged exposure to 40°C is 15 h, two hours less than the time taken to reach the first peak after a short exposure to high temperature.

A further point which is particularly illustrated by the data presented in Fig. 3.30, is that the phase shift produced by a 4-h 40°C treatment is not stable; the magnitude of the shift becomes either greater or smaller on successive peaks. This is due to the lengthening of the period following the 40°C treatment and will be discussed more fully later.

The results presented in Figs 3.26 - 3.30 do not establish which of the phase shifts represent phase delays and which represent phase advances. In an attempt to establish the direction of the induced phase shifts leaves were subjected to 40°C for only 1 h. The magnitude of the phase shift which occurred was then compared with that induced by a 4-h exposure to 40°C applied so that it ended at the same time in the cycle as the 1-h treatment. The 1-h 40°C treatment would be expected to give rise to a smaller phase shift than the 4-h treatment and thus provide clear evidence as to whether the phase had been advanced or delayed. These high temperature treatments were applied only in those positions in the cycle where a 4-h treatment had previously been shown to give rise to a large phase shift.

The results of four such experiments are shown in Figs. 3.31 - 3.34 and the collated data in Fig. 3.35. The dark and light continuous lines in Figs. 3.31 - 3.34 represent the rhythms in leaves subjected to 40°C for 4 h and 1 h respectively. The control rhythm in leaves held continuously at 15°C is shown by the broken lines. For the reason already mentioned, the phase shift induced by a 40°C treatment is not stable, thus, where figures given for the magnitude of the phase shift are discussed they refer to the phase shift produced on the first peak following the treatment.

FIGURE 3.31. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* in light and normal air at 15°C by exposure to 40°C for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at 0600 h as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (0200 h) and the second arrow the time at which the 1-h treatment began (0500). The broken line represents the rhythm in untreated control leaves. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.31

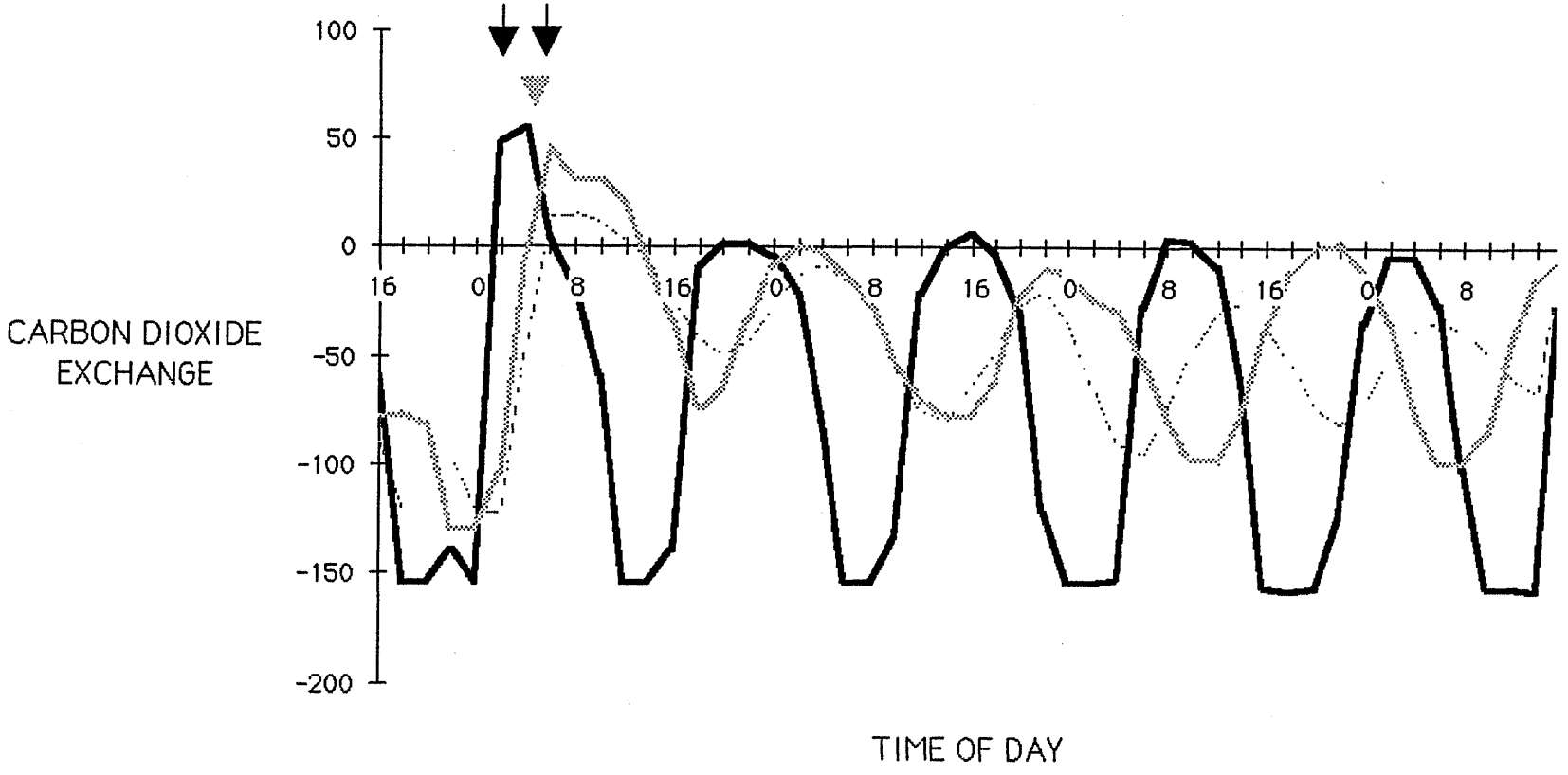

100
50
0
-50
-100
-150
-200
16
0
8
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.32. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to 40°C for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at 0800 h as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (0400 h) and the second arrow the time at which the 1-h treatment began (0700). The broken line represents the rhythm in untreated control leaves. Presentation as for Fig. 3.31.

FIGURE 3.32

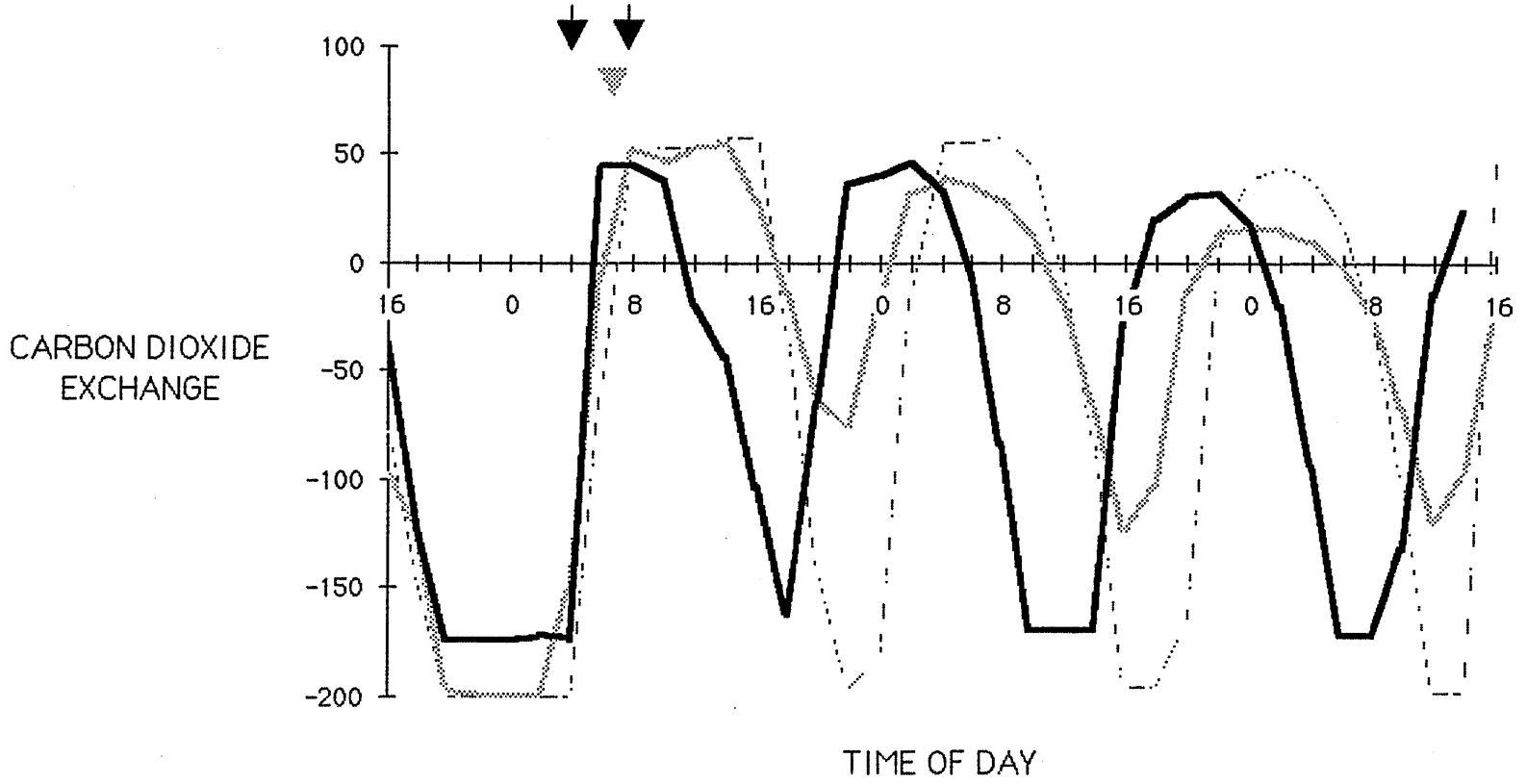

CARBON DIOXIDE EXCHANGE
100
50
0
-50
-100
-150
-200
16
0
8
16
0
8
16
0
8
16
TIME OF DAY

FIGURE 3.33. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to 40°C for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at 2100 h as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (1700 h) and the second arrow the time at which the 1-h treatment began (2000). The broken line represents the rhythm in untreated control leaves. Presentation as for Fig. 3.31

FIGURE 3.33

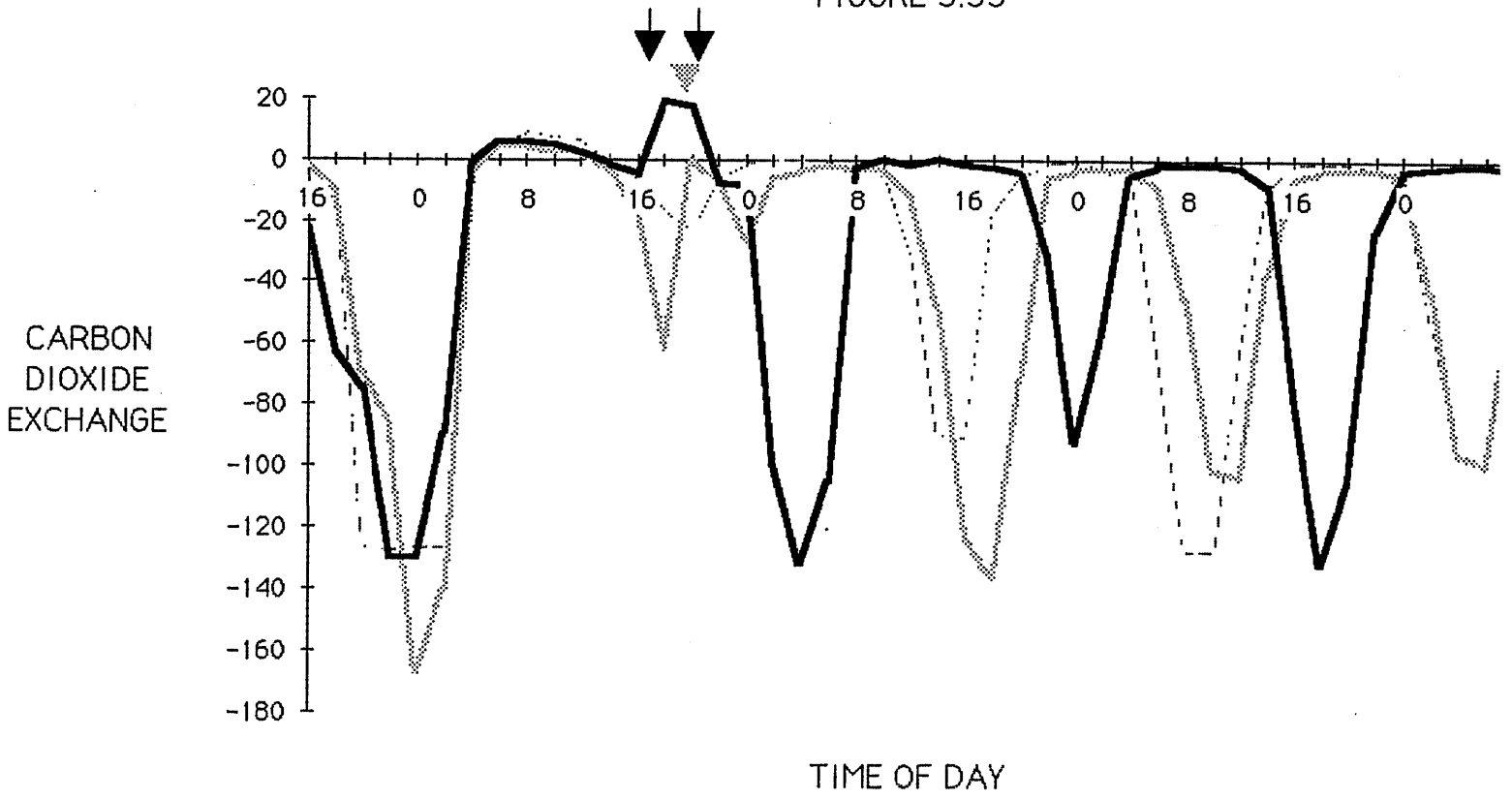
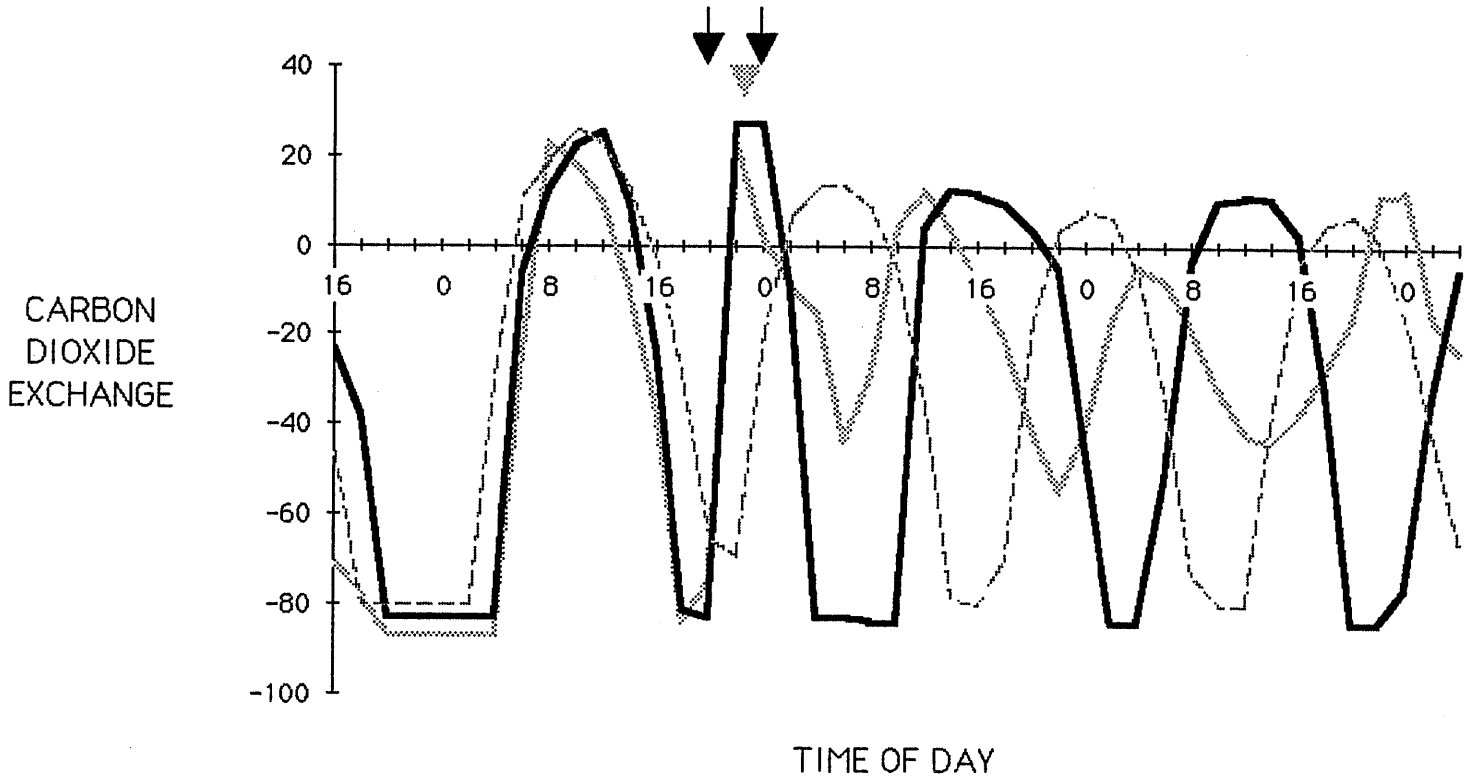

20
0
-20
-40
-60
-80
-100
-120
-140
-160
-180
16
0
8
16
0
8
16
0
8
16
0
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.34. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to 40°C for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at midnight as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (2000 h) and the second arrow the time at which the 1-h treatment began (2300 h). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.31.

FIGURE 3.34

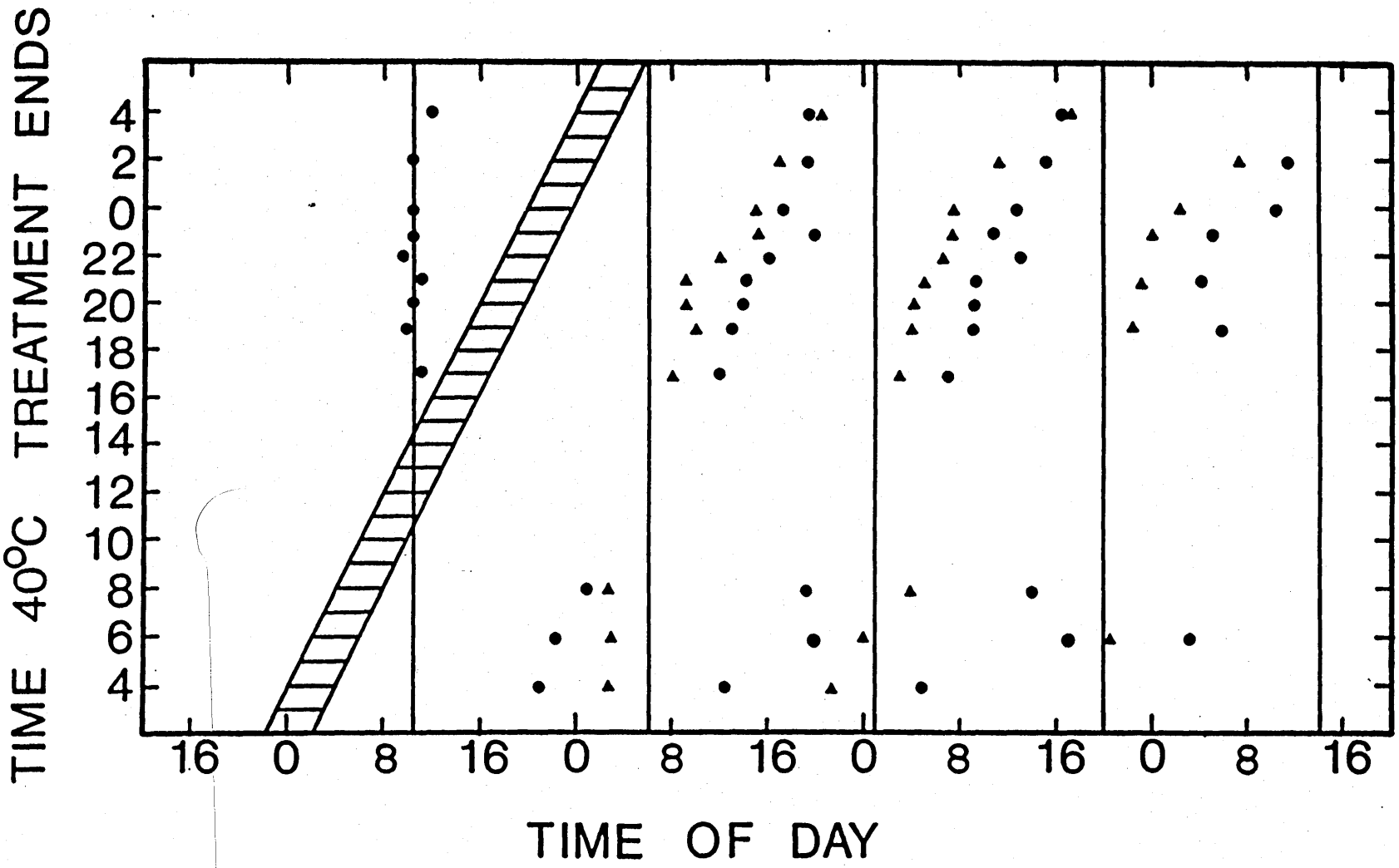

CARBON
DIOXIDE
EXCHANGE
40
20
0
-20
-40
-60
-80
-100
16
0
8
16
0
8
16
0
8
16
0
TIME OF DAY

In Fig. 3.31, the 1-h and 4-h treatments were applied from 0500 h to 0600 h and from 0200 to 0600 h respectively, ending approximately 4 h before the usual time of occurrence of the first peak of the control rhythm. The 1-h high temperature treatment advances the phase of the following peak by only 1 h whilst the 4-h treatment shifts the phase of this peak by approximately 6 h. When the 40°C treatement is applied so that it ends at 0800 h on the second day of the experiment a phase advance also appears to occur. The results of this experiment are shown in Fig. 3.32. The 4-h treatment advances the phase of the rhythm by approximately 6 h whilst the shorter 1-h treatment induces an advance of only 2 h. In contrast, the phase of the rhythms in CO_2 exchange is delayed in response to 1- and 4-h exposures to 40°C ending at 2100 h in the trough of the rhythm as shown in Fig. 3.33. A phase delay of approximately 9 h is induced by the 4-h treatment, and one of about 3 h by the 1-h treatment. In Fig. 3.34 the 1- and 4-h treatments ended at midnight, approximately 5 h before the occurrence of the 2nd peak of the control rhythm. At this position in the cycle the 4-h treatment delayed the occurrence of the first post-treatment peak by about 11 h and the 1-h treatment by approximately 7 h.

The collated data in Fig. 3.35 indicate that phase delays are induced by exposing leaves to 40°C for either 1 or 4 h at any time in the cycle between 1700 h on the second day of the experiment and 0200 h on the third day. In other words, a short duration 40°C treatment delays the phase of the rhythm when the treatment ends a few hours before the occurrence of the second trough in the rhythm until a few hours before the occurrence of the third peak. Exposing leaves to 40°C between 0400 h and 0800 h before the occurrence of the first peak, however, appears to advance the phase. These findings may indicate that the state of the oscillator a few hours before the occurrence of the first and second peak in the rhythm is different.

It was mentioned previously that exposing leaves to 40°C for 4 h appears to

FIGURE 3.35. Collated data for a series of experiments in which the cycle of CO_2 exchange in *Bryophyllum* leaves was scanned with a 4-h (●) or 1-h (▲) treatment during which time the temperature was increased to 40°C. Leaves were otherwise maintained in continuous light and normal air at 15°C. Times of occurrence of the peaks of untreated control rhythms are represented by the vertical lines. Points in any horizontal line show the mean times of the occurrence of the peaks of the rhythms in two samples of leaves following a treatment at the time indicated on the ordinate. Treatment times are indicated by the shaded bar.


TIME 40°C TREATMENT ENDS
4
2
0
22
20
18
16
14
12
10
8
6
4
16
0
8
16
0
8
16
0
8
16
0
8
16
TIME OF DAY

affect the period of the subsequent rhythm at 15°C. Considering firstly only the experiments in which both samples of leaves were exposed to 40°C for 4 h (i.e. Figs. 3.26 - 3.30), the mean period of the rhythm was 19.6 ± 0.12 h, which is significantly longer than the mean period of 18.5 ± 0.15 h recorded in control leaves held continuously at 15°C. This finding was confirmed in experiments designed to determine the direction of the phase shift (i.e Figs. 3.31 - 3.35). In these experiments the average period in leaves exposed to 40°C for either 1 or 4 h was 19.1 ± 0.12 h and 19.2 ± 0.30 h respectively, both of which are significantly longer than the period of 18.3 ± 0.17 h recorded in control rhythms monitored during these experiments. Since the rhythm following a high temperature treatment was only monitored for up to 4 cycles, it is possible that the increase in period length following a high temperature treatment may reflect a transient instability.

The increase in period length has consequences for studies of phase shifting. At points in the cycle where a high temperature treatment causes a phase advance, the advance on successive peaks will become progressively smaller. Exactly the opposite effect will be observed when treatments result in a phase delay; the delay on successive peaks will become progressively larger. The results presented in Figs. 3.30 and 3.35 do give some indication that this occurs.

The effects of decreasing the temperature from 15 to 2°C for 4 h at four different points in the cycle are shown in Figs. 3.36 - 3.39 and the results of a large number of such experiments in which the cycle was scanned at hourly intervals are illustrated diagrammatically in Fig. 3.40. Although two samples of leaves were treated with 2°C in each experiment, the curve of only one sample is shown because the rhythms overlapped to too great an extent to allow both to be illustrated clearly.

In Fig 3.36 the temperature was decreased from 15 to 2°C for 4 h from 1600 h to 2000 h in a trough in the rhythm and no phase shift resulted. The next peak occurred approximately 7 h after the end of the low temperature treatment and

FIGURE 3.36. The effect of exposing leaves of *Bryophyllum fedtschenkoi* to 2°C for 4 h from 1600 h to 2000 h as indicated by the arrows. Leaves were otherwise maintained in continuous illumination and a stream of normal air at 15°C. The rhythm in the treated leaves is represented by the continuous line and that in untreated control leaves by the broken line. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (fresh\ weight)^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.36

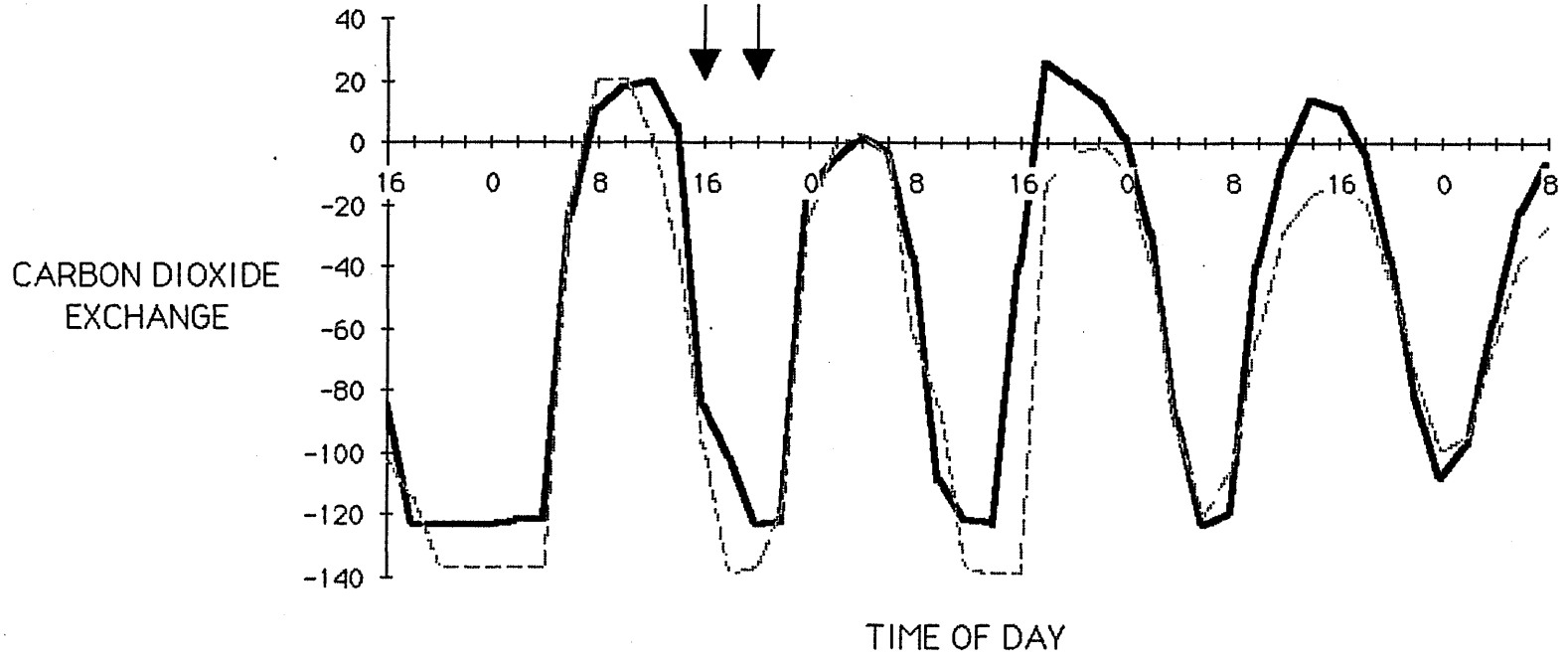

CARBON DIOXIDE EXCHANGE
40
20
0
-20
-40
-60
-80
-100
-120
-140
16
0
8
16
0
8
16
0
8
16
0
8
TIME OF DAY

FIGURE 3.37. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination and a stream of normal air at 15°C by exposure to 2°C for 4 h from midnight to 0400 h as indicated by the arrows (continuous line). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.36.

FIGURE 3.37

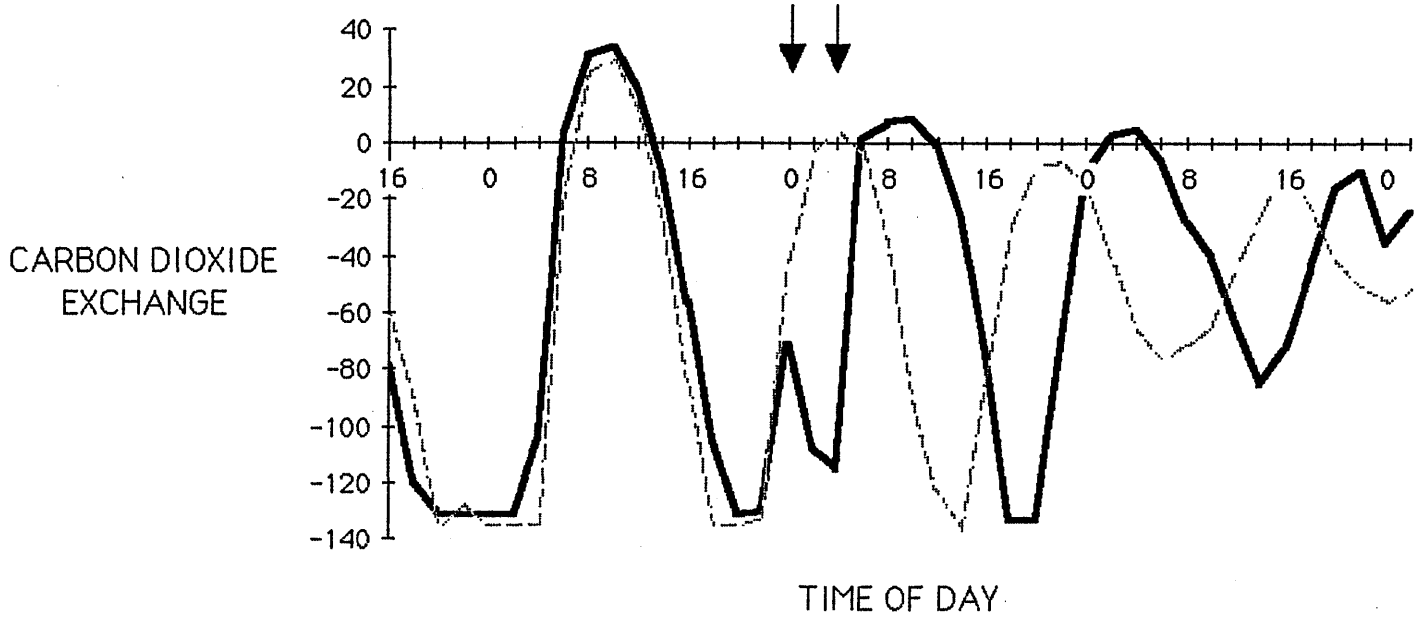

CARBON DIOXIDE EXCHANGE
40
20
0
-20
-40
-60
-80
-100
-120
-140
16
0
8
16
0
8
16
0
8
16
0
TIME OF DAY

FIGURE 3.38. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination and a stream of normal air at 15°C by exposure to 2°C for 4 h from 0800 h to midday as indicated by the arrows (continuous line). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.36.

FIGURE 3.38

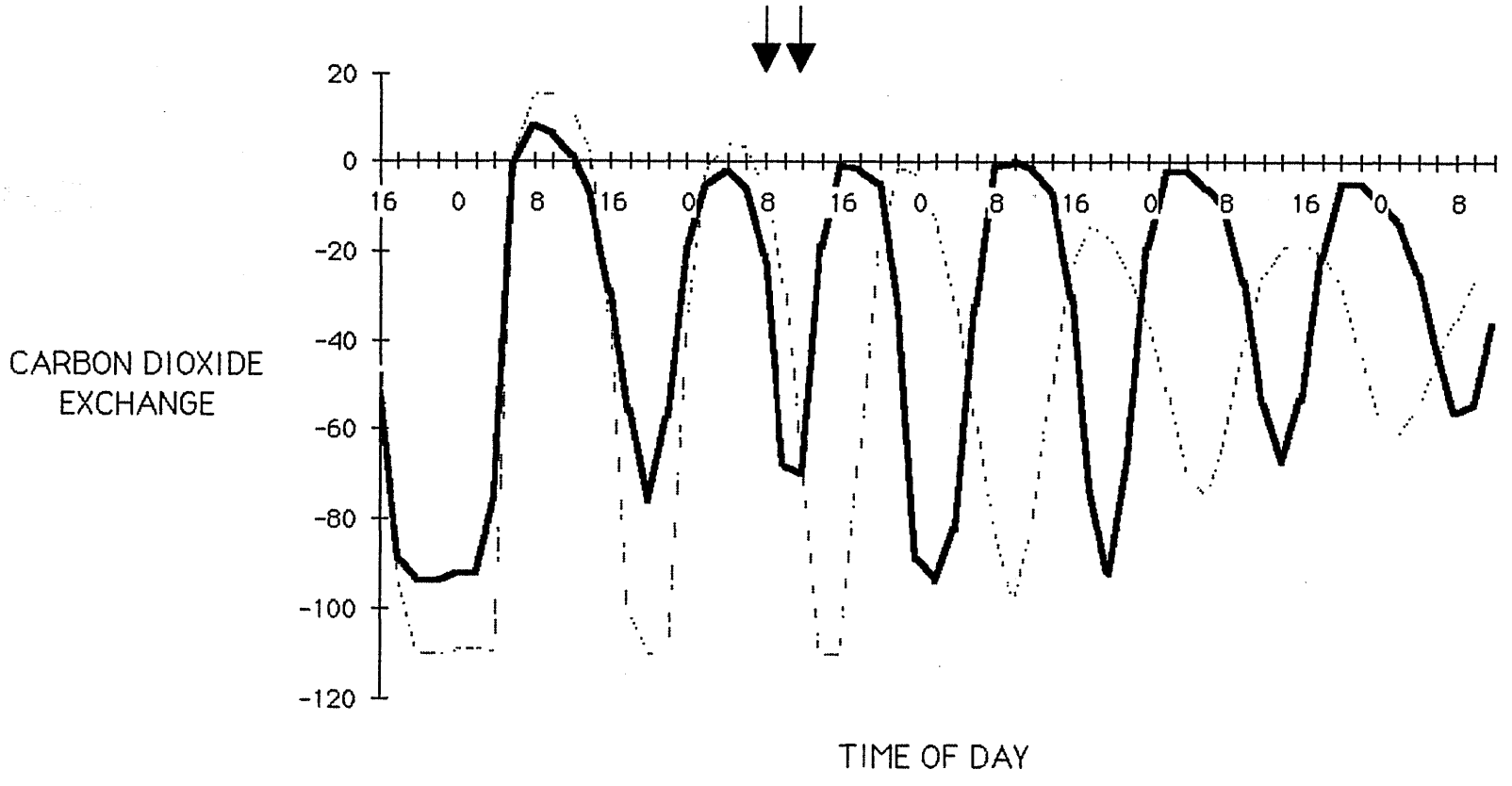

20
0
-20
-40
-60
-80
-100
-120
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.39. The effect of exposing leaves of *Bryophyllum fedtschenkoi* to 2°C for 4 h from midday to1600 h as indicated by the arrows (continuous line). The broken line represents the rhythm in untreated control leaves. Leaves were otherwise maintained in continuous illumination and a stream of normal air at 15°C. Presentation as for figure 3.36.

FIGURE 3.39

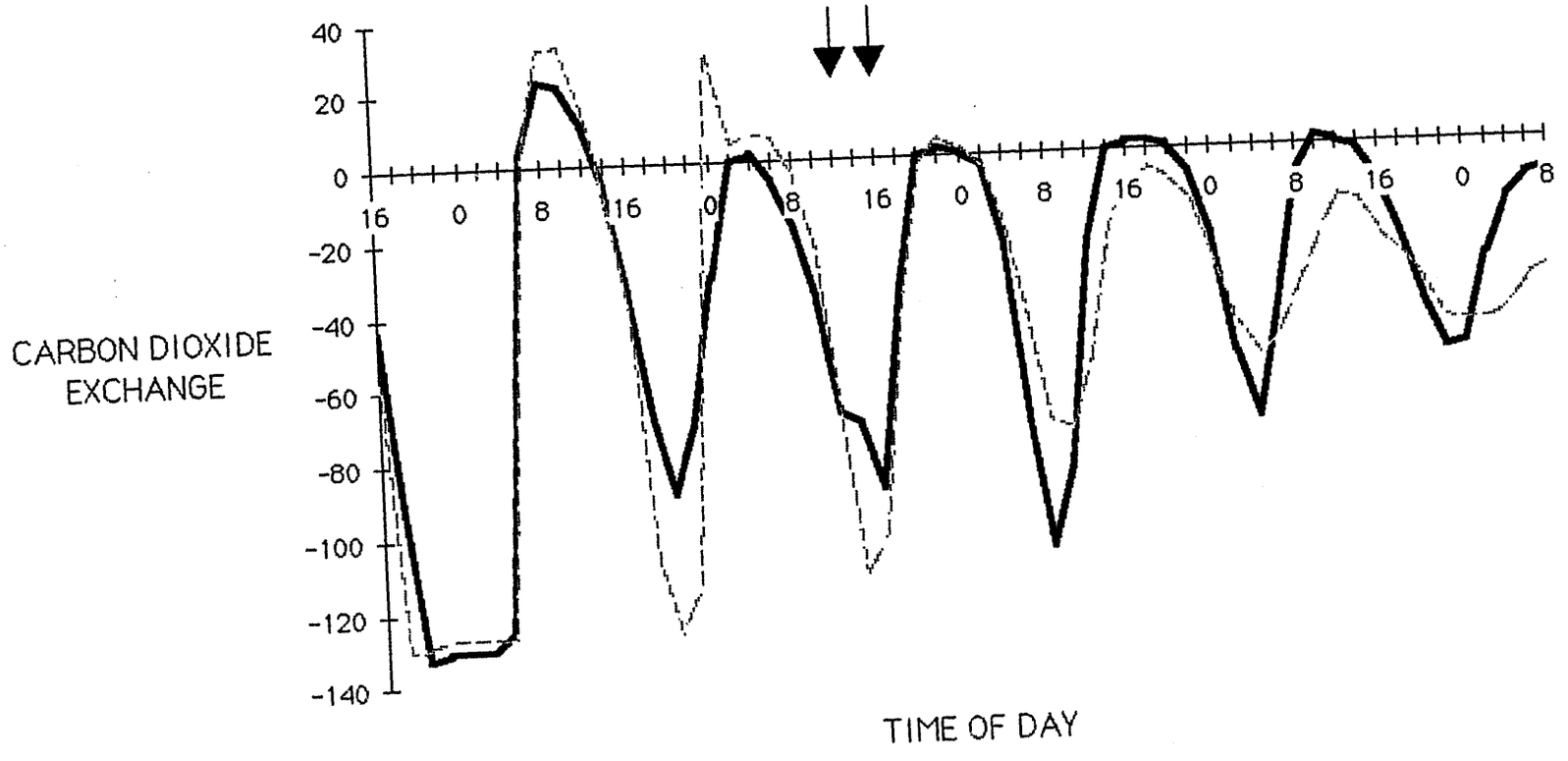
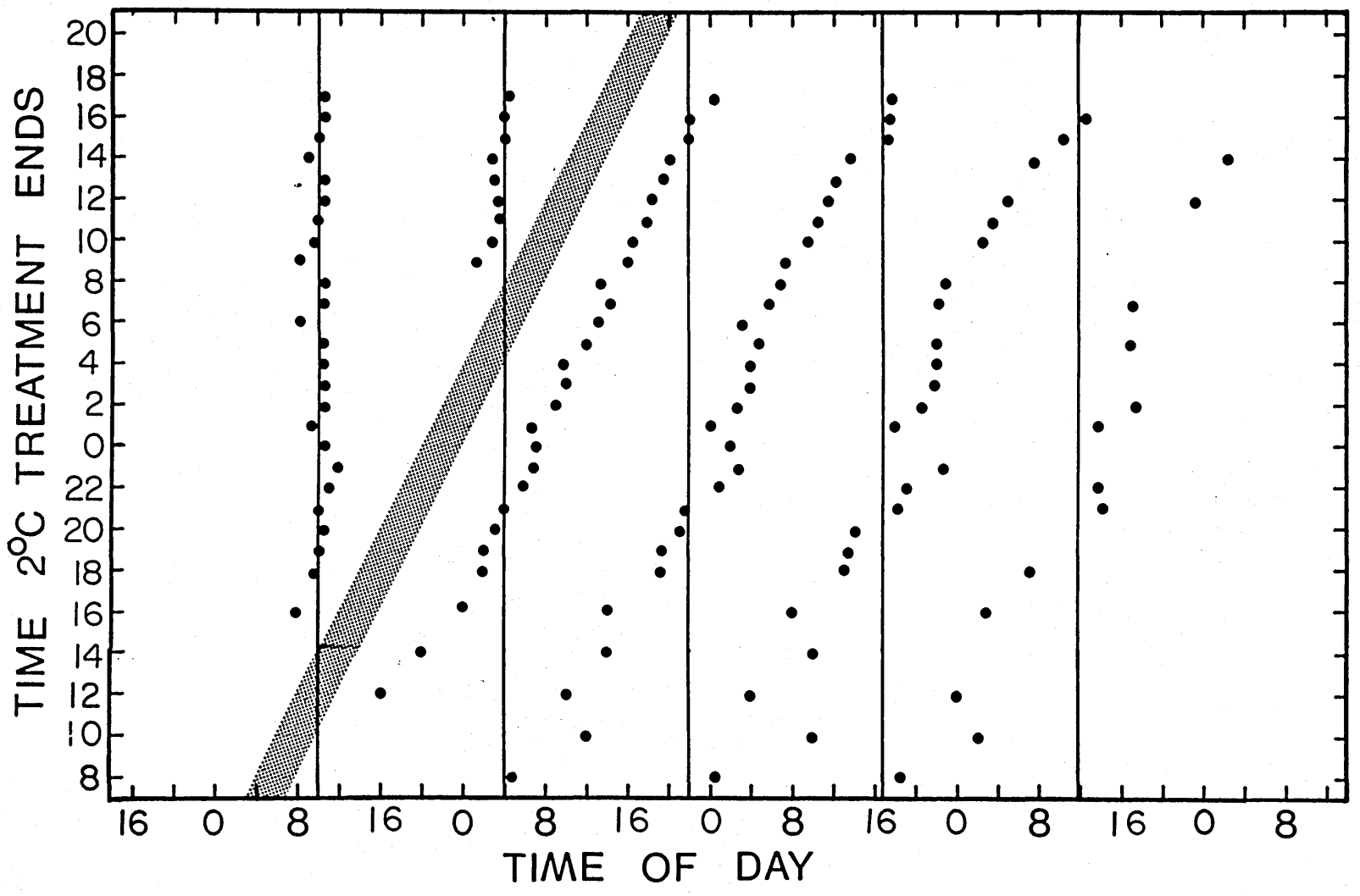

CARBON DIOXIDE EXCHANGE
40
20
0
-20
-40
-60
-80
-100
-120
-140
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
TIME OF DAY

FIGURE 3.40. Collated data for a series of experiments in which the cycle of CO_2 exchange in *Bryophyllum* leaves was scanned at approximately hourly intervals with a 4-h treatment during which time the temperature was decreased to 2°C. Leaves were otherwise maintained in continuous light and a stream of normal air at 15°C. Times of occurrence of the peaks of untreated control rhythms are represented by the vertical lines. Points in any horizontal line show the mean times of occurrence of the peaks in the rhythms of two samples of leaves following a treatment at the time indicated on the ordinate. Treatment times are indicated by the shaded bar.


TIME 2°C TREATMENT ENDS
20
18
16
14
12
10
8
6
4
2
0
22
20
18
16
14
12
10
8
16 0 8 16 0 8 16 0 8 16 0 8 16 0 8
TIME OF DAY

subsequent peaks at intervals of approximately 17 - 18 h. A large phase shift was induced when the low temperature treatment was applied from midnight to 0400 h (Fig. 3.37). When the temperature was lowered there was an increase in the rate of CO_2 uptake but after the treatment ended the rate of uptake decreased rapidly resulting in a peak of the rhythm approximately 6 h later. A phase shift was also induced by a low temperature treatment ending at noon on the third day of the experiment, as shown in Fig. 3.38. Decreasing the temperature from 12 midday to 1600 h in a trough in the rhythm on the second day of the experiment was not effective in inducing a phase shift, the first peak occurred 7 h after the low temperature treatment ended and coincided with the peak in the control rhythm (Fig. 3.39).

The nature of the phase shift induced by a 4-h exposure to $2^{o}C$ is shown in Fig. 3.40. The magnitude of the phase shift is clearly determined by the time in the cycle at which the low temperature treatment ends. Irrespective of the time of day at which the low temperature treatment is applied, the next peak always occurs approximately 7 h after the end of the treatment. The simplest interpretation of this result is that low temperature induces a phase shift in the rhythm by forcing the basic oscillator to, and holding it at, a fixed phase point in the cycle. Such a view would be consistent with that formed in the previous section to account for the inhibition of the rhythm caused by prolonged exposures to low temperature. However, the time taken for the first peak to occur when the leaves are returned to $15^{o}C$ after a prolonged $2^{o}C$ treatment is about 4 h whereas after a 4-h treatment it is about 7 h.

In an attempt to establish whether the phase shifts shown in Fig. 3.40 were advances or delays, leaves were subjected to $2^{o}C$ for only 1 h and the magnitude of the phase shift was compared with that induced by a 4-h treatment as previously described for high temperature treatments.

The dark and light continuous lines in Figs. 3.41 - 3.44 are 4 examples of the

FIGURE 3.41. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to 2°C for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at 1000 h as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (0600 h) and the second arrow the time at which the 1-h treatment began (0900 h). The broken line represents the rhythm in untreated control leaves. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g$ (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.41

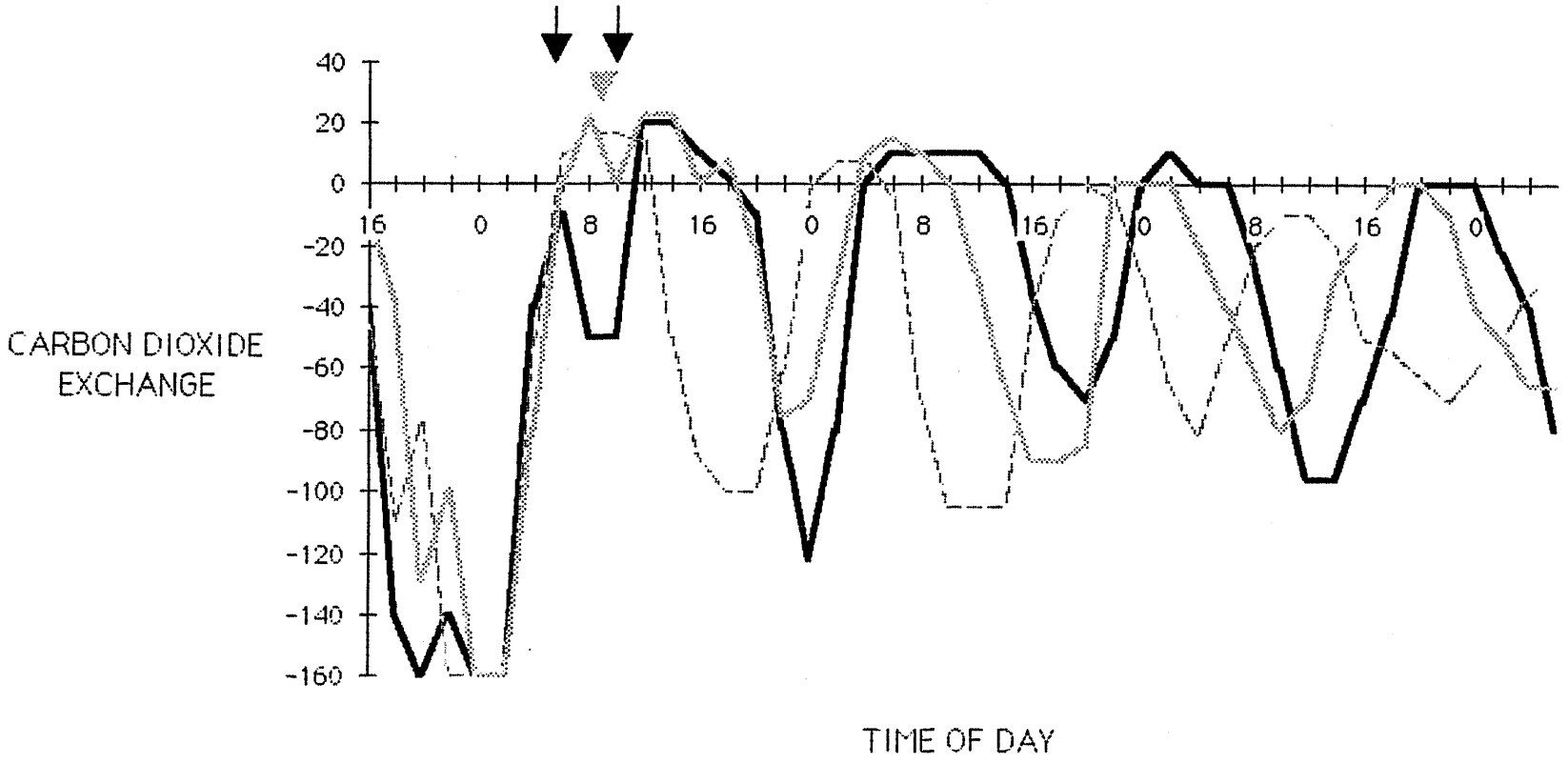

CARBON DIOXIDE EXCHANGE
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
16
0
8
16
0
8
16
0
8
16
0
TIME OF DAY

FIGURE 3.42. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to 2°C for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at midday as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (0800 h) and the second arrow the time at which the 1-h treatment began (1100 h). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.41.

FIGURE 3.42

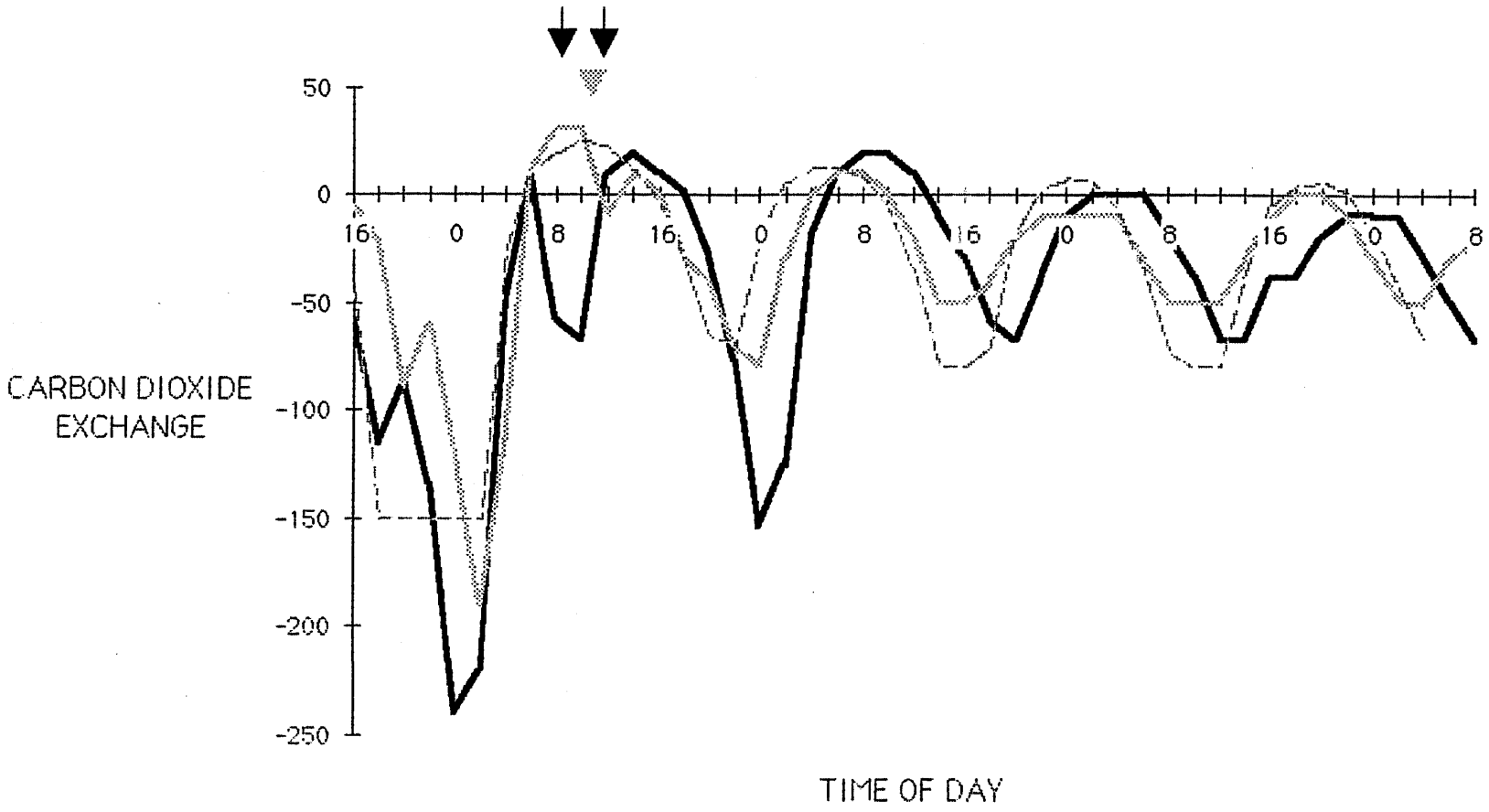

50
0
-50
-100
-150
-200
-250
16
0
8
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.43. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to 2°C for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at 1300 h as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (0900 h) and the second arrow the time at which the 1-h treatment began (midday). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.41.

FIGURE 3.43

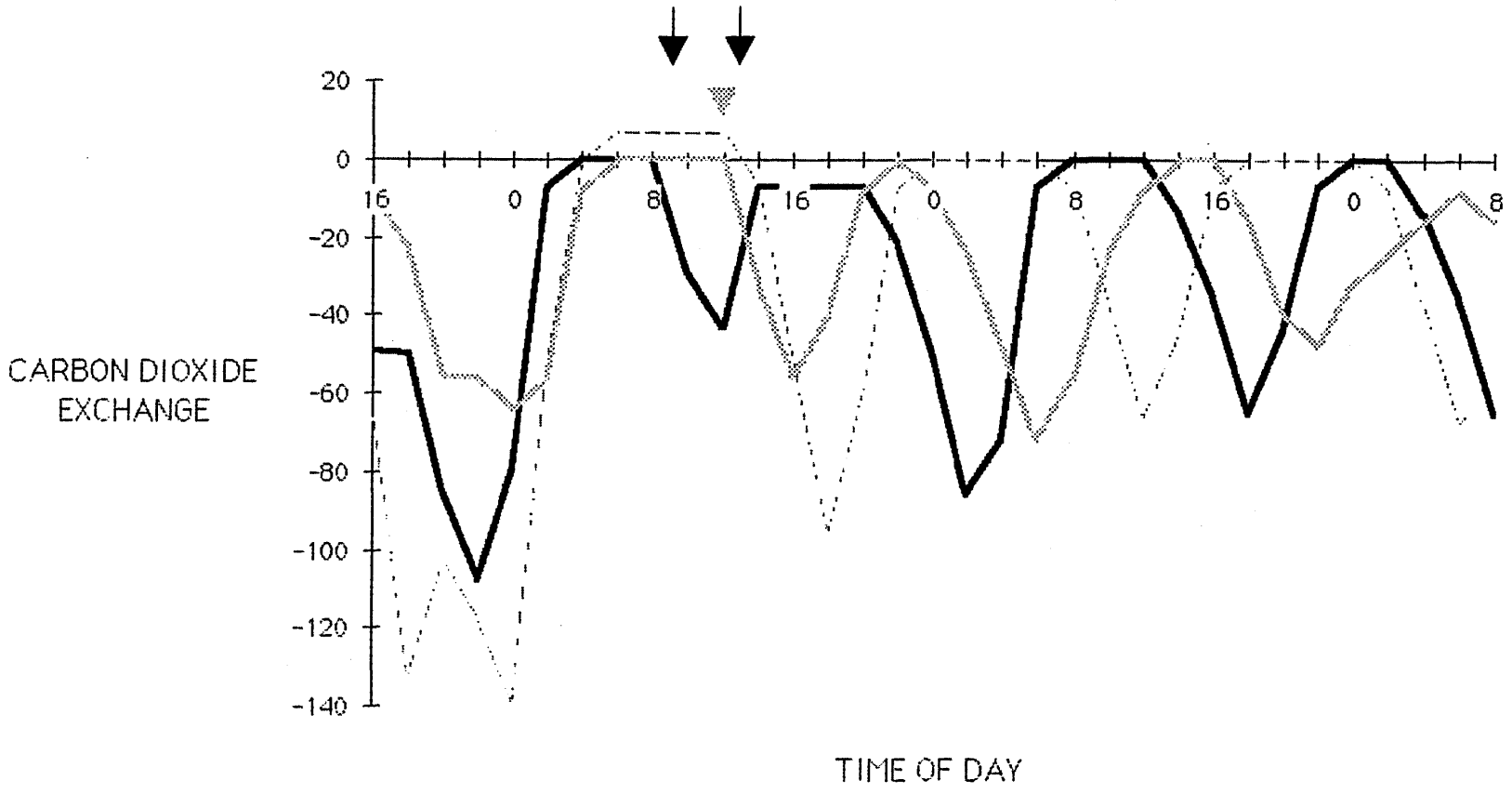

20
0
-20
-40
-60
-80
-100
-120
-140
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.44. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to 2°C for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at 1500 h as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (1100 h) and the second arrow the time at which the 1-h treatment began (1400 h). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.41.

FIGURE 3.44

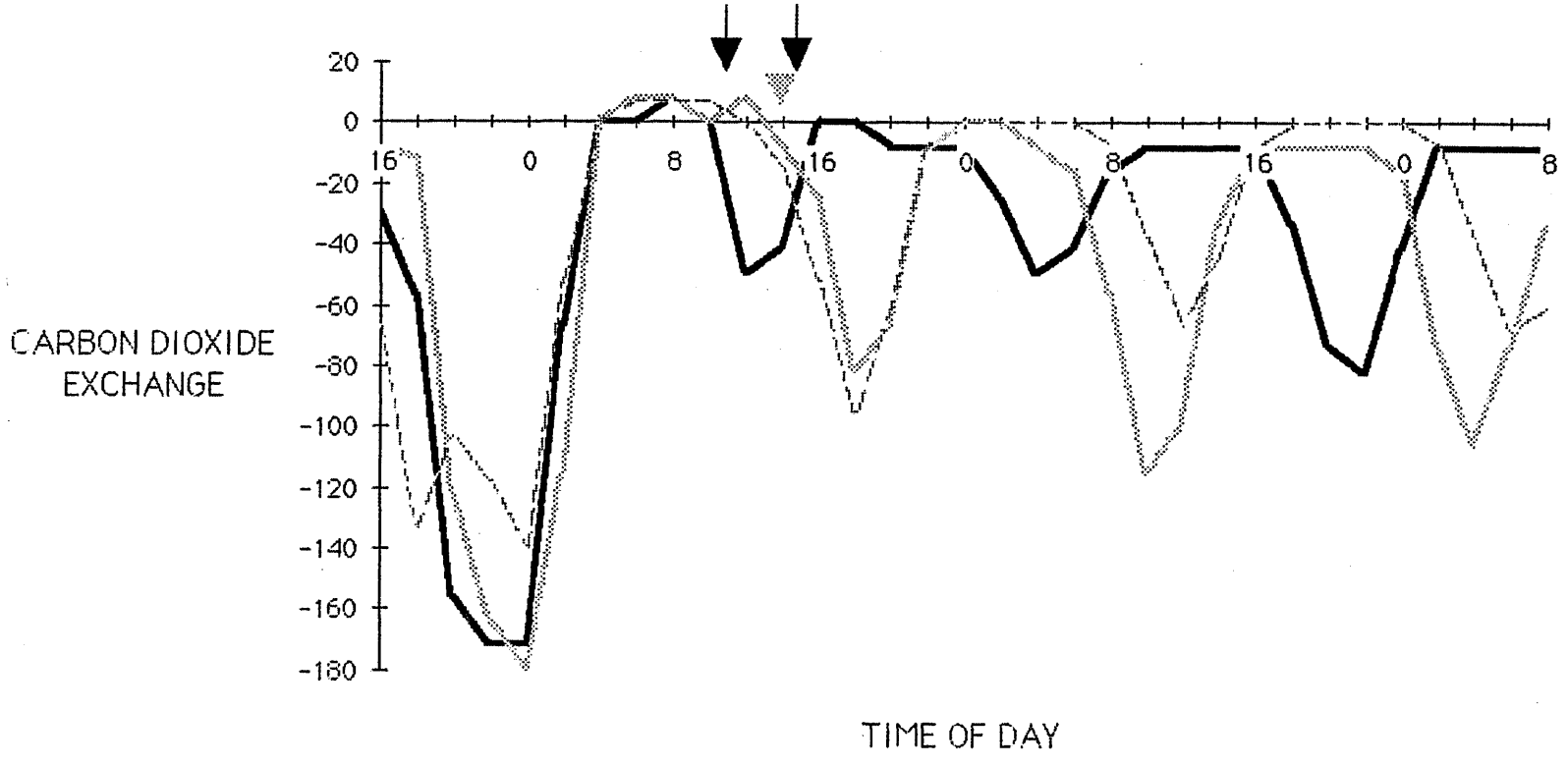
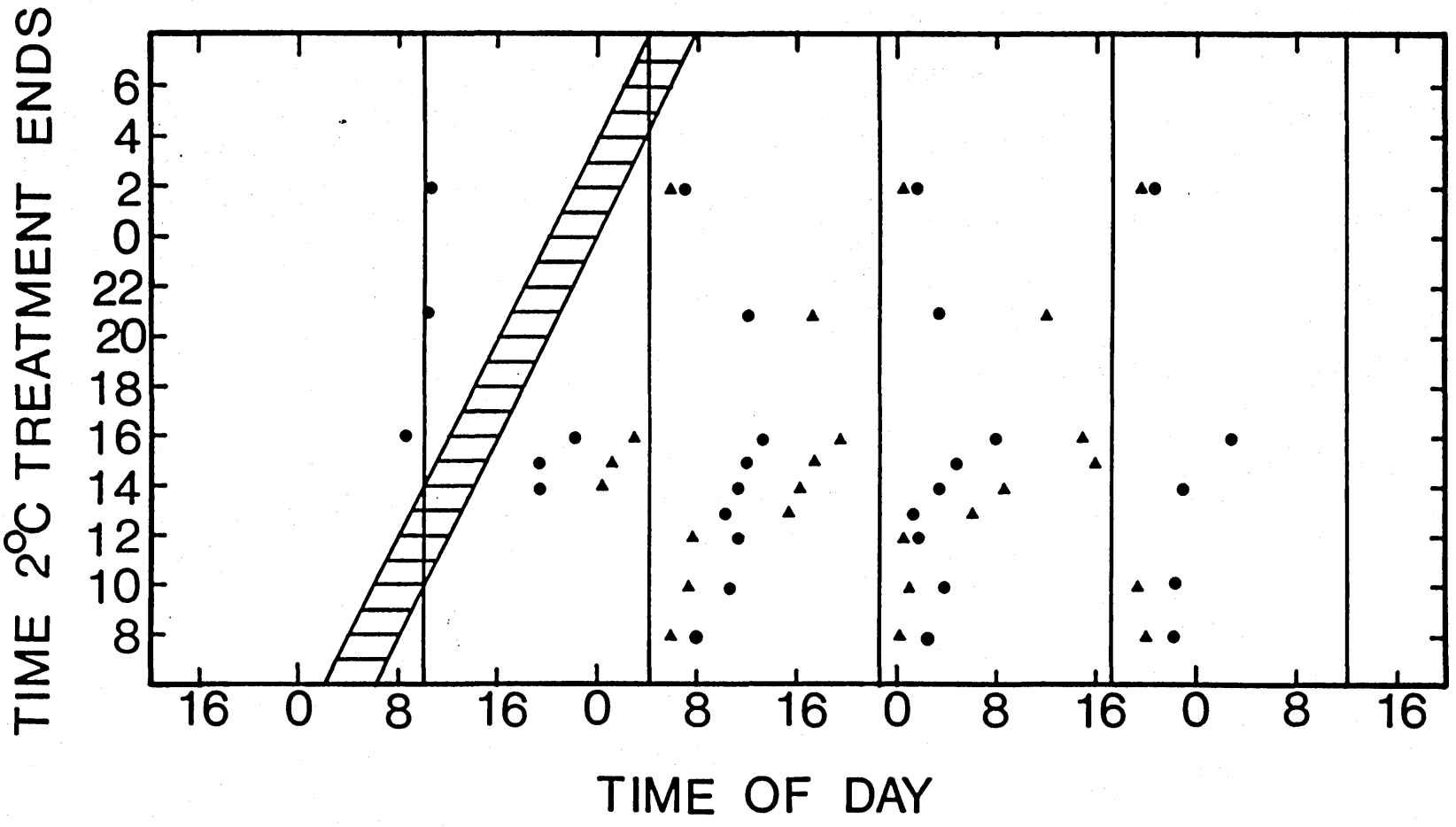

20
0
-20
-40
-60
-80
-100
-120
-140
-160
-180
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.45. Collated data for a series of experiments in which the cycle of CO_2 exchange in *Bryophyllum* leaves was scanned with a 4-h (●) or 1-h (▲) treatment during which time the temperature was raised to 2°C. Leaves were otherwise maintained in continuous light and normal air at 15°C. Times of occurrence of the peaks of untreated control rhythms are represented by the vertical lines. Points in any horizontal line show the mean times of the occurrence of the peaks of the rhythms in two samples of leaves following a treatment at the time indicated on the ordinate. Treatment times are indicated by the shaded bar.


TIME 2°C TREATMENT ENDS
6
4
2
0
22
20
18
16
14
12
10
8
16
0
8
16
0
8
16
0
8
16
0
8
16
TIME OF DAY

results obtained when leaves were exposed to 2°C for 4 h and 1 h respectively at four different positions in the cycle. In all experiments the 1- and 4-h low temperature treatments ended at the same time. The control rhythms in leaves kept at 15°C are shown by the broken lines. Results of a whole series of such experiments are shown in Fig. 3.45.

Exposing leaves to 2°C for 1 h from 0900 h to 1000 h, and for 4 h from 0600 h to 1000 h, at the top of the first peak in the rhythm delays the phase. The 1-h treatment shifted the phase of the following peak by approximately 2 h and the 4-h treatment by approximately 4 h (Fig. 3.41). A phase delay also appears to occur in response to 1- and 4-h 2°C treatments ending at midday on the second day of the experiment, approximately 1 - 2 h after the occurrence of the first peak. The 4-h treatment delays the phase of the following peak by approximately 4 h while the 1-h treatment induces a delay of only 1 h. These results are shown in Fig. 3.42. In Fig. 3.43 the 1- and 4-h low temperature treatments ended at 1300 h, 2 - 3 h after the first peak of the rhythm. In this position in the cycle, the advance on the next peak induced by the 1- and 4-h treatments was approximately 6 and 10 h respectively. Advances of approximately 2 and 6 h were induced by 1- and 4-h treatments respectively ending at 1500 h (Fig. 3.44).

Considering the collated data in Fig. 3.45, it appears that phase delays are induced by short duration low temperature treatments ending between 0800 h and midday on the second day of the experiment, times which correspond approximately to 2 h before and after the occurrence of the first peak in the rhythm. Exposing leaves to low temperature for either 1 or 4 h between 1300 h and 1700 h advances the phase of the rhythm. The crossover point between phase advances and phase delays would therefore seem to be at 1300 h, 2 - 3 h after the time of occurrence of the first peak. It cannot be assumed from these results that treatments ending at similar positions before and after the second peak of the rhythm would shift the phase in a similar direction, particularly in view of the results obtained for high

temperature (Fig. 3.40). As expected from the above results a 4-h treatment ending at 0200 h on the third day of the experiment, about 3 h before the occurrence of the second peak, resulted in a phase delay. The validity of this result is, however, questionable due to the close similarity in the magnitude of the phase shift induced by a 1- and 4-h treatment. A particularly interesting point to be revealed by these results is that 2°C treatments ending at 0800 h and 1000 h on the second day of the experiment resulted in phase delays whilst 40°C treatments applied at these positions induced phase advances.

Exposure to low temperature for 4 h also led to a modification of the period. In contrast to high temperature treatments however, which appear to slow down the oscillator, low temperature treatments appear to speed up the frequency of oscillation when the leaves are returned to 15°C. The average period of the rhythm at 15°C in leaves following a 4-h, 2°C treatment, calculated from the experiments in which two samples of leaves were treated simultaneously (Figs. 3.36 -3.40), is 17.6± 0.15 h, a value significantly shorter than the period of control leaves maintained continuously at 15°C (18.4 ± 0.16 h). This finding is supported by the periods obtained in experiments designed to determine the direction of the phase shifts induced by low temperatures (Figs. 3.41 - 3.45). The mean periods in leaves following a 4-h and 1-h exposure to low temperature are 17.2 ± 0.24 h and 17.4 ± 0.18 h respectively, both of which are significantly shorter than the period of 18.3 ± 0.18 h recorded in the control rhythm at 15°C in these experiments. Since the rhythm was monitored for up to 4 cycles only after the temperature change, the observed speeding up of the oscillation is probably caused by a transient decrease in the length of the period. It is of considerable interest, however, that high and low temperatures have the opposite effects on the period. High temperature appears to slow the oscillator while low temperature appears to speed it up. Attention is drawn to section 3.1.1.1. where it was reported that the period of oscillation increases with increasing ambient temperature.

Shortening of the period as a result of a brief exposure to low temperature also has implications for phase shifting. Shorter periods will result in a gradual increase in the amount of phase advance produced on successive peaks. On the other hand, when the $2^{o}C$ treatment ends at a point in the cycle where a delay is induced, the delays will become progressively smaller on successive peaks. Evidence for the occurrence of this phenomenon is provided in Figs. 3.40 & 3.45.

An important finding to emerge from the results presented in this section is that the oscillator is sensitive to low temperature in those parts of the cycle in which it is insensitive to high temperature. Exposing leaves to $40^{o}C$ for 4 h at a peak in the rhythm induces little or no phase shift whilst an exposure to $2^{o}C$ in this position induces a large phase shift. In contrast, a 4-h exposure to $40^{o}C$ in the trough of a rhythm produces a large phase shift but a $2^{o}C$ treatment given in this position has no effect on the phase. These findings are entirely consistent with those reported in the previous section where it was found that prolonged exposure to high and low temperature inhibited oscillation by driving the oscillator to, and holding it at, fixed phase points in the cycle which differed by 180^{o}.

In order to clarify the direction of phase shifts induced by high and low temperature it would be necessary to scan at least one complete cycle with 4 - and 1-h temperature treatments. Nevertheless, it appears that low temperature delays the phase of the rhythm at times in the cycle at which high temperature advances the phase. Similarly low temperature advances the phase of the rhythm in those positions in the cycle where high temperature delays the phase. Thus the effects of high and low temperature are opposite with respect to both the magnitude and direction of the phase shifts induced.

Since high temperature had previously been found to inhibit the rhythm by holding leaves in a malate-poor state, and low temperatures by holding the leaves in a malate-rich state, it seemed reasonable to predict that short exposures to high and low temperatures may exert their effect on the phase by altering the malate

status of the leaves. The next step in this investigation therefore aimed to establish whether or not this prediction was correct by comparing the malate status in leaves held at 15°C for a specific duration with that in leaves exposed to 40 or 2°C for 4 h at a time in the cycle where such treatments had previously been shown to result in a large phase shift.

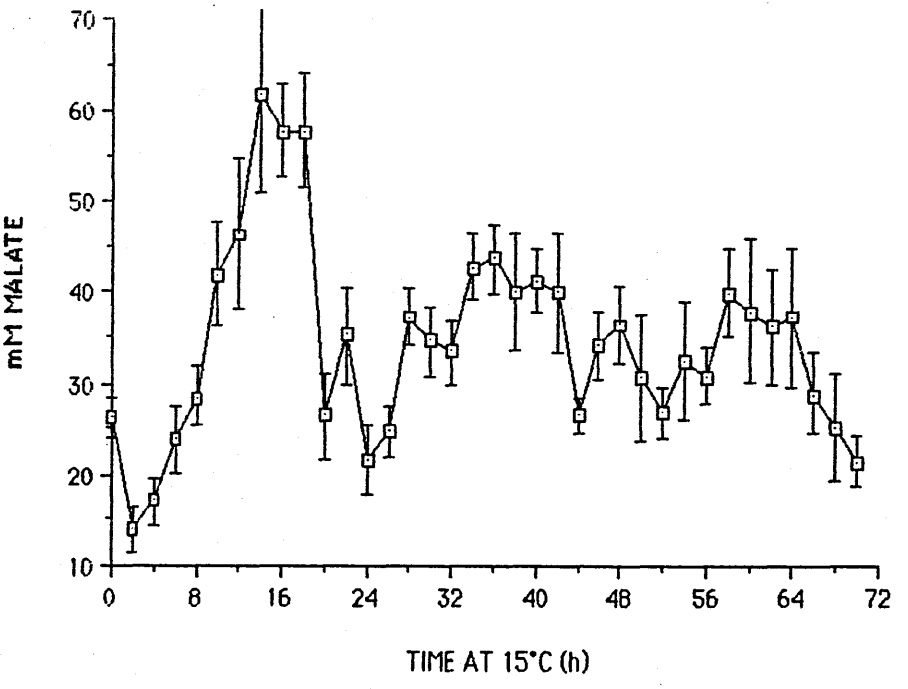
First, however, the malate status of leaves maintained in a stream of normal air and at 15°C was monitored during the first 72 h of the rhythm in an attempt to establish if the rhythm in CO_2 exchange observed at this temperature was accompanied by a rhythm in the malate status of the leaves. The results of this investigation are shown by the curves in Figs. 3.46 A and B.

The curve in Fig. 3.46A represents the average results of 3 independent experiments. In each experiment the malate concentration in the sap of two leaves was measured separately every two hours throughout the cycle. The concentration of malate in the sap of individual leaves was found to be somewhat variable, and it was appreciated that slight differences in the phase of the rhythms in individual leaves would obscure any rhythm in malate content, therefore a further two experiments were carried out in which an attempt to minimise these variabilities was made by using more leaves for each measurement. The results of these experiments are shown in Fig. 3.46B where the curve represents the average results of two experiments; in each the malate content of 6 leaves was assayed individually every 4 h for 70 h. Thus, each point on the curve represents the mean value of 12 leaves.

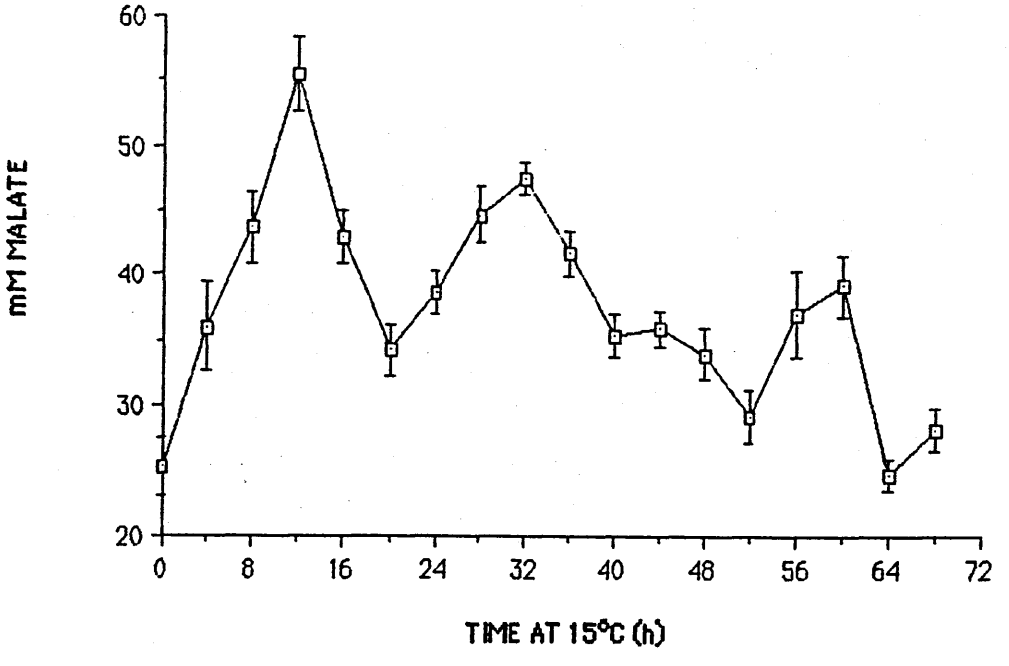
The results, shown in Figs. 3.46A & B indicate that there is indeed an oscillation in the malate content of leaves held in constant conditions. This rhythm is, however, less pronounced than the rhythm of CO_2 exchange monitored under the same conditions and, in contrast to the gas exchange rhythm, which persists for 10 - 14 days, the malate rhythm appears to damp out after only two cycles. In Fig 3.46C

FIGURE 3.46 A & B. Concentration of malate (mM) in the extracted cell sap of leaves held in continuous light and a stream of normal air at 15°C. Each point on the curve in Fig 3.46 A represents the mean concentration of malate in the sap of 6 leaves and in 3.46 B each point on the curve represents the mean concentration of malate in the extracted cell sap of 12 leaves. The vertical lines represent ± S.E. of the mean values. Ordinate: concentration of malate(mM). Abscissa: time at 15°C in hours.

A


mM MALATE
70
60
50
40
30
20
10
0
8
16
24
32
40
48
56
64
72
TIME AT 15°C (h)

B


mM MALATE
60
50
40
30
20
0
8
16
24
32
40
48
56
64
72
TIME AT 15°C (h)

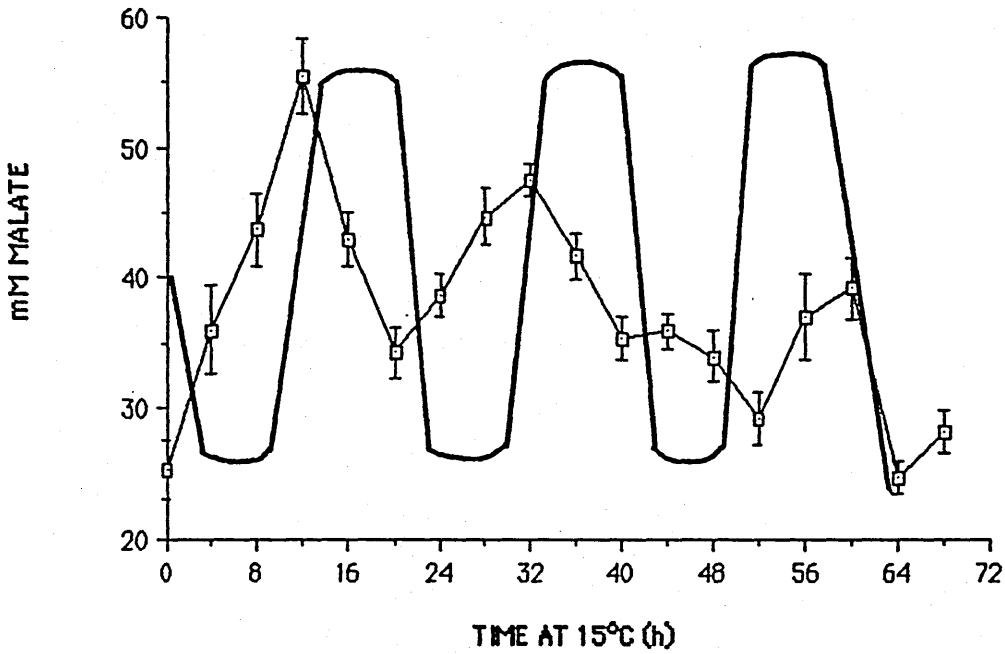


FIGURE 3.46C. Relationship between the concentration of malate in the extracted cell sap of leaves maintained in continuous illumination and a stream of normal air at 15°C (□) and the pattern of CO_2 exchange in leaves under these conditions (continuous line). Presentation as for Figs. 3. 46 A & B. For explanation see text.

a typical CO_2 curve has been drawn overlying the rhythm in malate content shown in Fig. 3.46B. The values of the CO_2 exchange rhythm are arbitrary; this curve has been plotted merely to show the relationship between the times of occurrence of the peaks and troughs in the CO_2 exchange and the malate rhythm. The concentration of malate in the extracted cell sap increases from approximately 25 mM at the beginning of the experiment to a maximum concentration of 55 - 60 mM 12 - 14 h later. The maximum malate levels are therefore attained approximately 4 - 6 h before the average time of occurrence of the first peak in the CO_2 exchange rhythm. The malate concentration then decreased reaching a minimum value of 25 - 35 mM 20 - 24 h after the leaves were placed at 15°C, approximately 4 - 6 h before the occurrence of a trough in the CO_2 exchange rhythm. The next maximum in malate concentration occurred approximately 20 h after the first, and was approximately 2 - 3 h earlier than the corresponding peaks in the CO_2 exchange rhythm. The time of occurrence of the third peak in the malate content of the leaves was difficult to assess but it appeared to occur approximately 24 h after the second, some 2 h after the usual time of occurrence of the third peak in the rhythm of CO_2 exchange.

It should be noted that the time in the cycle at which the malate content of the leaves is at its second maximum corresponds approximately with the time at which a high temperature treatment results in a large phase shift and a low temperature treatment induces only a small shift. Furthermore, the first minimum in the malate content of the leaves corresponds approximately to a time in the cycle at which a high temperature treatment results in only a small phase shift whilst a low temperature treatment induces a large phase shift.

The pH of the leaf cell sap used for the malate determinations presented in Fig. 3.46B was measured and the results are presented in Fig. 3.47. These results indicate

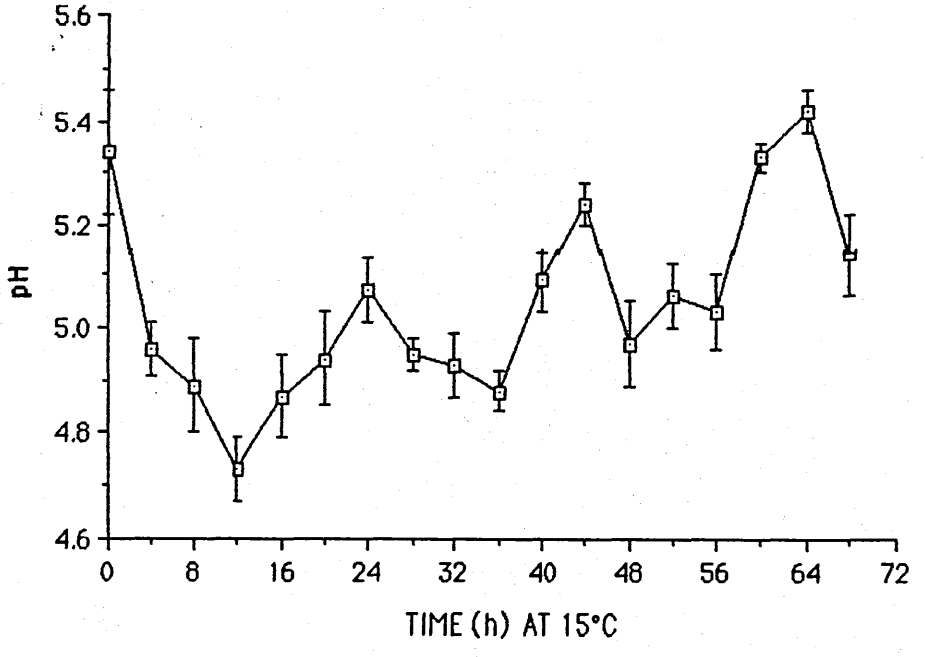


FIGURE 3.47. The pH of the extracted cell sap of leaves maintained in continuous light and a stream of normal air at 15°C. Ordinate: pH. Abscissa: Time at 15°C in hours.

that the pH of the cell sap oscillates with a period of approximately 20 h. The pH decreased from 5.3 at the beginning of the experiment to 4.6 12 h later, a time which corresponds with the time at which the malate content of the leaves has reached a maximum value. A reasonably good correlation was also observed between the malate content of the leaves and the occurrence of the second minimum in the pH value. After approximately 40 - 50 h in constant conditions however the relationship between the malate content of the leaves and the pH is difficult to establish because the times of occurrence of both the third peak in malate content and the third peak in the pH are difficult to assess.

The results presented in Figs. 3.46A and B show that the malate levels in leaves maintained in continuous light at 15°C oscillate with a period of approximately 18 - 20 h for at least two days. Whether or not this rhythm is related to, and can account for, the rhythm of CO_2 exchange recorded under the same conditions is uncertain.

The next question to be addressed was whether or not the phase shifting effects of short exposures to either high or low temperature could be explained in terms of those treatments altering the malate status of the leaves.

In order to investigate the effects of a 4-h 40°C treatment on the malate status of the leaves, the malate content of leaves exposed to 40°C from 1800 h to 2200 h on the second day of the experiment was compared immediately after the end of the high temperature treatment with that of leaves maintained continuously at 15°C until 2200 h. Essentially the same procedure was followed to investigate the effects of a 4-h 2°C treatment on the leaf malate status, except that leaves were exposed to 2°C from 1000 h to 1400 h on the second day of the experiment. The reason for exposing leaves to 40 and 2°C at the times indicated above was that it had previously been established that large phase shifts were induced by 40 or 2°C treatments ending at these times. The results of two independent experiments and the average results of both experiments are presented in Tables 5 and 6. The data presented for each experiment represent the mean values of 6 leaves ± the S.E. of the means.

TABLE 5

COMPARISON OF THE LEVELS OF MALATE IN LEAVES EXPOSED TO 40°C FOR 4 h WITH THAT IN LEAVES KEPT AT 15°C.

	MALATE (mM) IN CELL SAP			
EXP NO.	CONTROL LEAVES	TREATED LEAVES	t	S
1	32.2 ± 1.67	27.5 ± 3.02	1.350	NS
2	37.3 ± 1.38	34.8 ± 2.57	0.833	NS
1+2	33.7 ± 1.04	31.5 ± 1.08	1.010	NS

TABLE 6.

COMPARISON OF THE LEVELS OF MALATE IN LEAVES EXPOSED TO 2°C FOR 4 h WITH THAT IN LEAVES KEPT AT15°C.

	MALATE (Mm) IN CELL SAP			
EXP NO.	CONTROL LEAVES	TREATED LEAVES	t	S
1	26.1 ± 2.83	30.0 ± 1.70	1.498	NS
2	24.3 ± 2.58	30.2 ± 2.2	1.812	NS
1 + 2	26.9 ± 2.00	27.3 ± 1.76	0.169	NS

It is evident from these results that exposing leaves to 40 or 2°C for 4 h does not significantly alter the gross malate status of the leaves. Nevertheless, it is

interesting to note that the malate concentration in the sap of leaves exposed to 40^0C was always lower whilst that in leaves exposed to 2^0C was always higher than the concentration in the control leaves kept continuously at 15^0C. This finding may imply that the high and low temperature treatments were not of sufficient duration to alter significantly the malate levels in the leaves. The CO_2 exchange pattern of the leaves used in the above experiments was monitored prior to extraction to check that the leaves were in a phase in the cycle at which either a 40 or 2^0C treatment would be expected to give rise to a large phase shift. In all experiments this was found to be the case. It is clear therefore that phase shifts induced by brief exposures to high and low temperature cannot be accounted for in terms of these treatments altering the gross malate status of the leaves.

3.2. THE EFFECTS OF DARKNESS ON THE RHYTHM OF CO_2 EXCHANGE

The rhythm of CO_2 exchange exhibited by leaves of *Bryophyllum fedtschenkoi* in a stream of normal air is inhibited by prolonged exposures to darkness (Wilkins, 1984). It has been suggested (Wilkins, 1984) that this inhibition is a consequence of malate accumulation in the leaf cells which inhibits further PEPCase activity. The leaves are unable to breakdown malate in continuous darkness, presumably because photosynthetic removal of the end products of breakdown is prevented. It seems possible, therefore, that darkness may inhibit the rhythm, and perhaps even control the phase, in a similar manner to low temperature which also appears to prevent malate breakdown. The aim of this section was to test this possibility. In particular it was hoped to establish:

1. The nature of the inhibition of the rhythm brought about by prolonged exposure of the leaves to darkness.
2. The malate status of leaves held in continuous darkness.
3. Whether or not short dark treatments shift the phase of the rhythm and, if so, whether the characteristics of the phase shifts are similar to those induced by low temperatures.

3.2.1. THE NATURE OF THE INHIBITION OF THE RHYTHM IN LEAVES HELD IN DARKNESS.

3.2.1.1. The Effects of Prolonged Exposure of the Leaves to Darkness

The nature of the inhibition of the rhythm in leaves maintained in normal air and darkness has been investigated by exposing leaves to darkness for a few days and then transferring them to continuous light at various times of day in order to establish whether or not a rhythm appeared, and if it did, whether the phase of the rhythm was related to the time of the transfer.

The continuous lines in Fig. 3.48 and 3.49 show the results of maintaining leaves in constant darkness for several days before transferring them to continuous light. The broken lines represent the rhythm in leaves held in continuous light throughout the experiment. The time of the transfer from continuous darkness (DD) to continuous light (LL) was midday in Fig. 3.48 and midnight in Fig. 3.49.

No rhythm was detected in leaves maintained in DD. During the first 8 - 10 h in DD leaves fixed CO_2, the maximum rate of fixation occurring at approximately midnight. After this time, the rate of CO_2 uptake decreased rapidly, net CO_2 uptake being replaced by net CO_2 output 6 h later. The rate of CO_2 output then increased to a value of approximately 20 - 30 $\mu g\ CO_2\ h^{-1}$ g (fresh weight)$^{-1}$, and thereafter either remained constant or decreased very gradually during the remainder of the time in DD. Net CO_2 fixation was never detected during this period. On transferring the leaves from DD to LL a rhythm appeared which persisted for at least 3 days with an apparently smaller amplitude than that of the control leaves which had been maintained throughout in continuous light. The leaves continued to give out CO_2 at a gradually decreasing rate for about 6 - 8 h after the transfer from DD to LL and the first peak occurred approximately 24 h after the end of the dark treatment regardless of the time of day at which the transfer was made. This point is illustrated by the collated data presented in Table 7 where the values represent the average time taken by at least two samples of leaves to reach the successive peaks in the rhythm after the end of a dark treatment.

FIGURE 3.48. Inhibition of the rhythm of CO_2 exchange in two samples of leaves of *Bryophyllum fedtschenkoi* held simultaneously in darkness, and its restoration on transferring the leaves to light (continuous lines). The broken line represents the rhythm in control leaves maintained in continuous light throughout the experiment. All samples of leaves were maintained in normal air at 15°C. The arrow indicates the time at which the leaves were transferred from dark to light (midday). Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.48

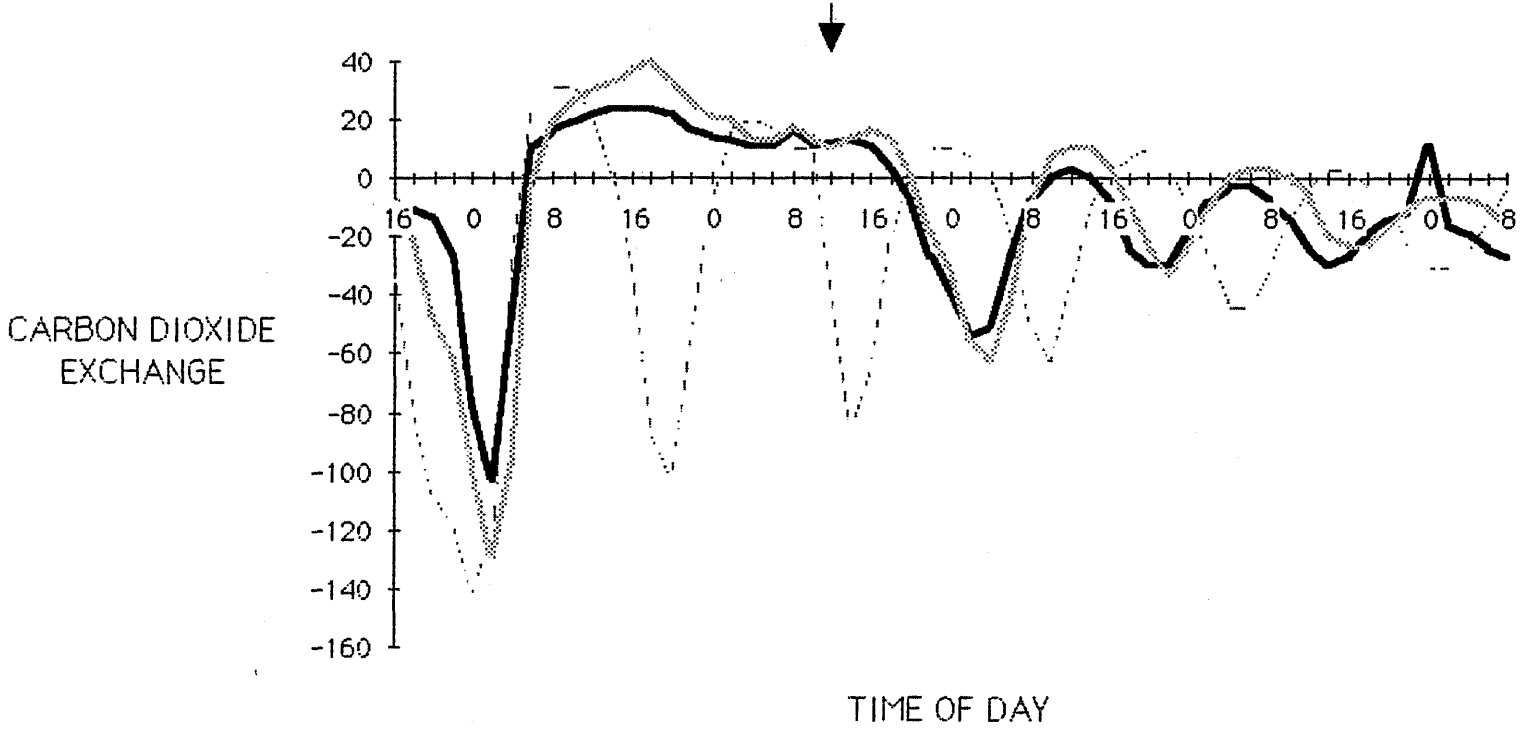

CARBON DIOXIDE EXCHANGE
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
TIME OF DAY

FIGURE 3.49. Inhibition of the rhythm of CO_2 exchange in two samples of leaves of *Bryophyllum fedtschenkoi* held simultaneously in darkness, and its restoration on transferring the leaves to light (continuous lines). The broken line represents the rhythm in control leaves maintained in continuous light throughout the experiment. All samples of leaves were maintained in normal air at 15°C. The arrow indicates the time at which the leaves were transferred from dark to light (midnight). Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.49

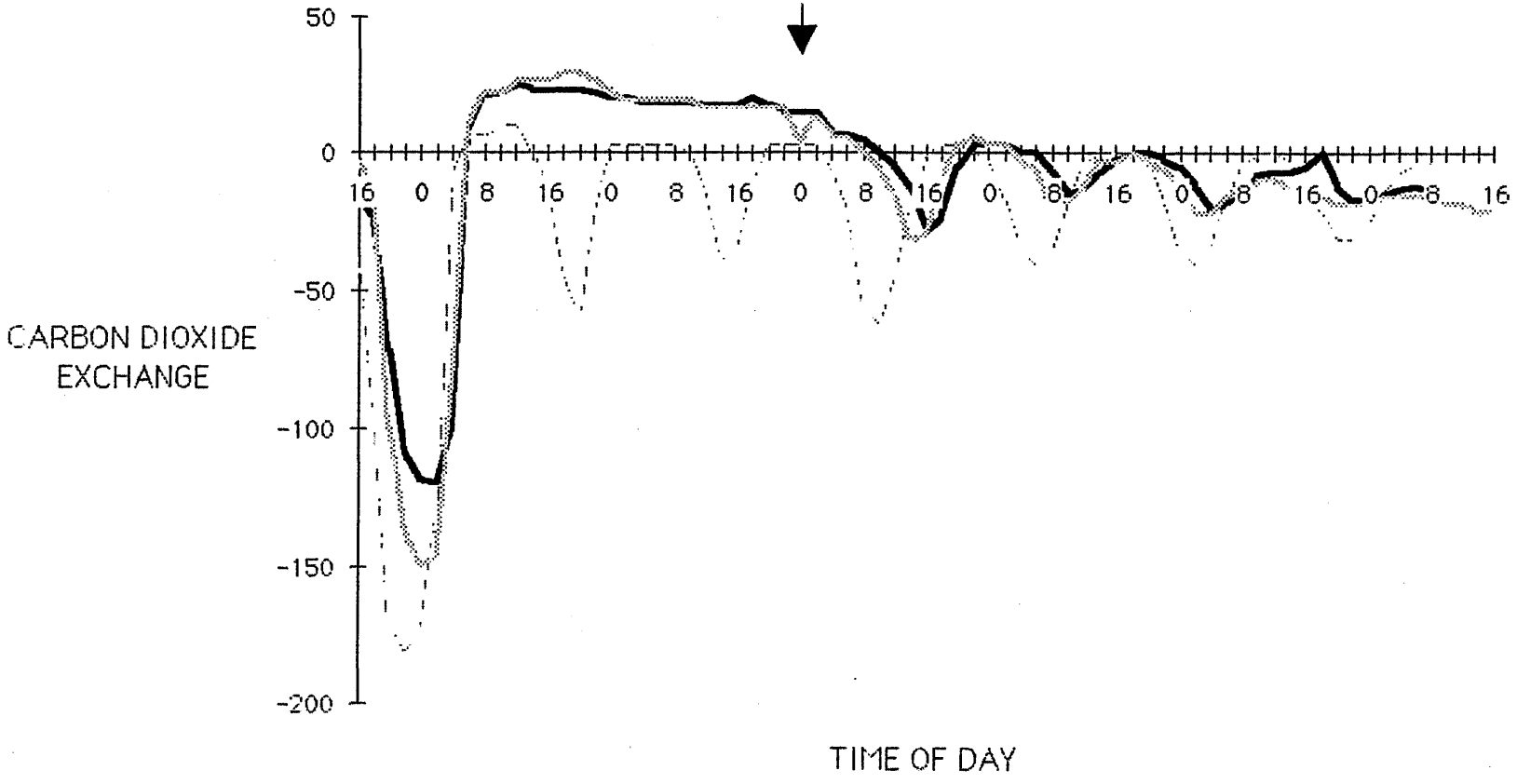

50
0
-50
-100
-150
-200
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
CARBON DIOXIDE EXCHANGE
TIME OF DAY

TABLE 7

TIME FROM END OF DARK TREATMENT TO OCCURRENCE OF PEAKS

TIME IN	TIME (h) TO:		
DARK (h)	1ST PEAK	2ND PEAK	3RD PEAK
38	24	42	59
44	24	43	60
56	25	43	61
80	24	41	58

These results indicate that the absence of a detectable rhythm in DD is a result of the oscillator being inhibited. If the oscillator had been operating, but the rhythm unable to manifest itself, then the fixed relationship between the end of the dark treatment and the phase of the newly initiated rhythm shown in Table 7 would not occur. Furthermore, the finding that the first peak of the rhythm always occurred a specific number of hours after the end of the dark treatment suggests that during the dark treatment the oscillator is held at a fixed phase point from which it is released on transferring the leaves to continuous light.

The initial period of CO_2 uptake which occurs during the first few hours in DD clearly indicates that CO_2 fixation is not inhibited by dark treatments. This period of fixation would presumably result in the accumulation of malate in the leaf cells. Malate removal would be prevented however, because photosynthesis is required to remove the end products of malate breakdown and the enzyme involved in this reaction may be inhibited by end products. The 6 - 8 h which elapse between transferring leaves from DD to LL and the occurrence of net CO_2 uptake may represent the time necessary for the leaf cells to remove sufficient amounts of malate to enable PEPCase to become active. These observations make it likely, therefore, that

darkness inhibits the rhythm in a similar manner to low temperature.

A further finding which illustrates the similarity of the effects of low temperature and darkness on the rhythm is the effect that darkness had on the length of the subsequent period in continuous light. The period of the rhythm in dark-treated leaves, 17.4 ± 0.20 h, is significantly shorter than the period of 19.3 ± 0.31 h recorded in control leaves maintained in continuous light during these experiments. Since the rhythm in dark-treated leaves was only monitored for up to three cycles it is possible that the difference in period length reflects only a transient instability of the period in dark-treated leaves. Futhermore, the period in the control leaves is somewhat longer than the period of 18 - 18.5 h usually recorded in leaves maintained in continuous light at 15^{0}C. This may merely reflect the fact that only a limited amount of data was available for determining the length of the periods in the above experiments.

If darkness inhibits the rhythm in a manner similar to that in which low temperature inhibits oscillation, then leaves maintained in DD would be expected to accumulate relatively large amounts of malate. This possibility was investigated by determining the malate status of leaves during the first 72 h in DD. The results of this investigation are presented in Fig. 3.50.

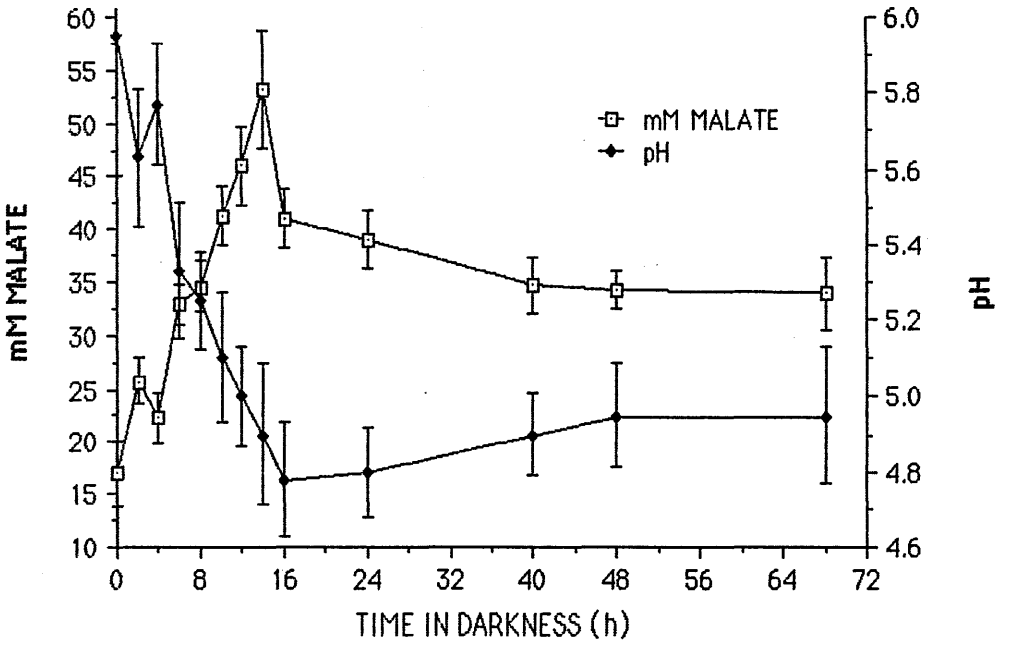


FIGURE 3.50. Concentration of malate and pH of the cell sap of leaves held in continuous darkness and a stream of normal air at 15°C. Each point on the malate and pH curves represents the average concentration of malate or the average pH respectively recorded in 6 leaves. The vertical lines represent ± the standard error of the mean values. Ordinate: mM malate (left), pH (right). Abscissa: time in continuous darkness (h).

These results show that very little change occurs in the malate concentration of the extracted cell sap during the first 4 h in DD. After this time the concentration of malate begins to increase and reaches a maximum value of approximately 50 mM 14 h after the beginning of the experiment. Thereafter the concentration of malate decreases somewhat reaching a constant value of approximately 35 - 40 mM between 24 and 40 h after placing the leaves in darkness. As predicted, darkness does lead to a state in which leaves contain relatively large amounts of malate. Nevertheless, it seems that the leaf cells are capable of removing a small amount of this malate because the concentration of malate determined in the sap from leaves held in darkness for 14 h was found to be significantly greater than that in leaves

which had been in darkness for 40, 48 and 68 h (t = 2.243, 2.307, 2.230 respectively). The changes in the malate content of the sap are also reflected in the pH curve. Very little change in the pH occurs during the first 4 h in darkness, but afterwards the pH begins to decrease and reaches a minimum of 4.8 16 h after placing the leaves in continuous darkness. For the next 24 h the pH appears to increase very slightly reaching a value of approximately 4.9 after 48 h in DD which remains constant for the remaining time in continuous darkness. This increase is not, however, significant. The pH after 16 h in DD was not found to be significantly different from that in leaves which had been in DD for 48 h (t = 1.089). Fig. 3.51 shows the CO_2 exchange pattern recorded in 3 samples of leaves during these experiments. The curves are similar to those shown in Figs. 3.48 and 3.49, the characteristics of which have already been described. It should be noted that the time at which CO_2 output reaches a constant value corresponds approximately with the time at which the concentration of malate in the cell sap reaches a constant value.

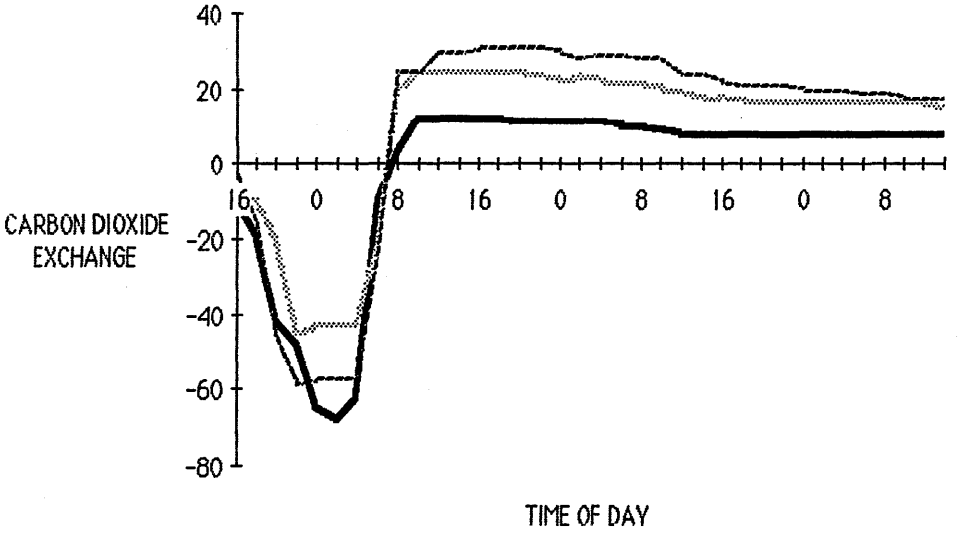


Figure 3.51. Inhibition of the rhythm of CO_2 exchange in three samples of leaves held in constant darkness and a stream of normal air at 15°C. Ordinate: the rate of CO_2 uptake (negative values) or output (positive values) in μg CO_2 h^{-1} g (fresh weight)$^{-1}$. Abscissa: time of day 0 = midnight.

3.2.1.2. The Effects of Pulse-Type Dark Treatments

The results presented in the previous section appear to indicate that prolonged exposure of the leaves to darkness inhibits the rhythm of CO_2 exchange in a similar manner to low temperature, that is, by leading to a state in which the leaf cells contain relatively large amounts of malate. It might be expected, therefore, that the phase of the oscillating system operating in continuous light would be sensitive to short exposures to darkness at those positions in the cycle at which it was found to be sensitive to exposures to low temperature. The next stage in the investigation therefore aimed to establish the precise phase points in the cycle at which the oscillator is sensitive to short dark treatments by subjecting leaves maintained in

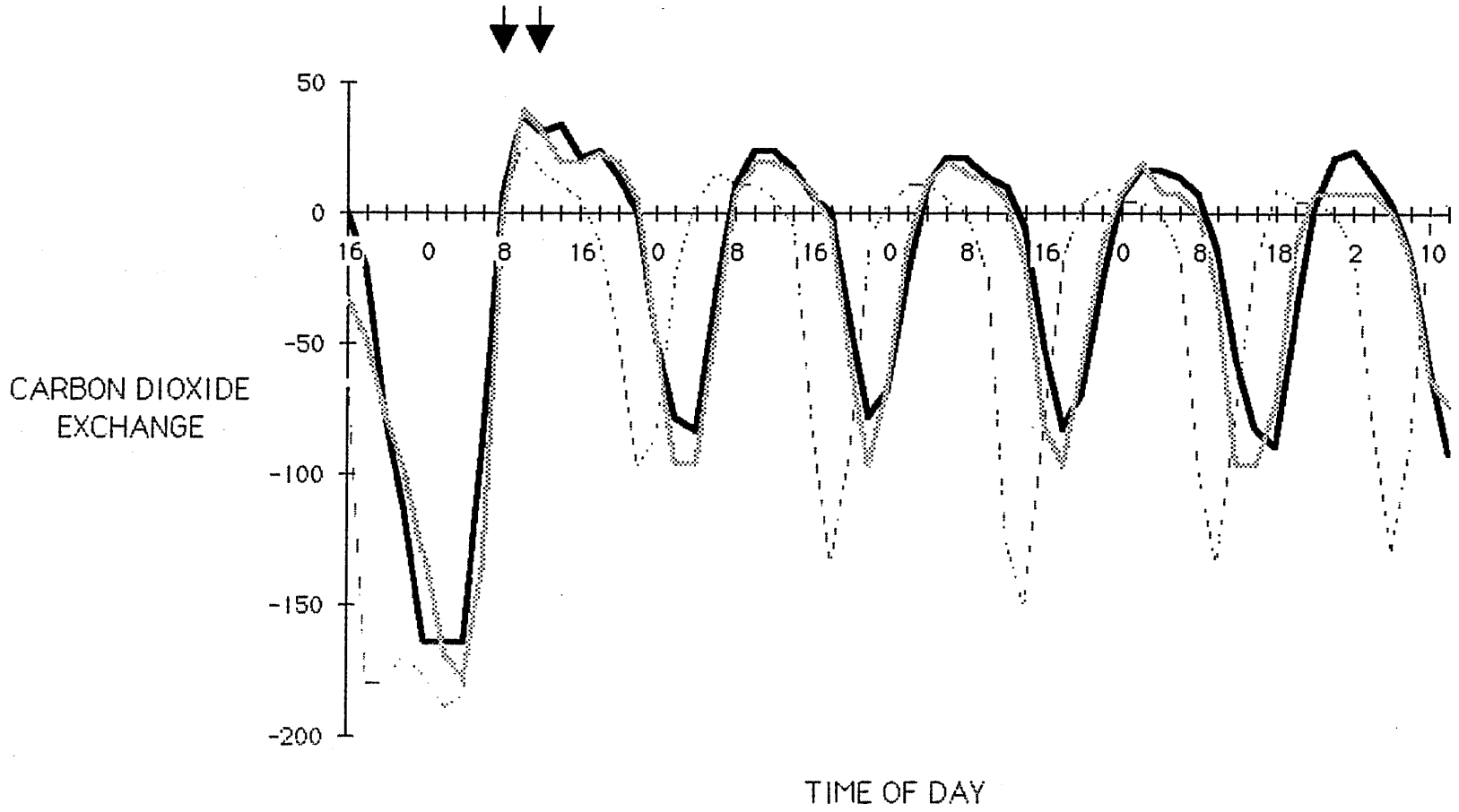
normal air and light at 15°C to darkness for 4 h at various positions in the cycle.

The continuous lines in Figs. 3.52 - 3.55 are representative of the results obtained when leaves were subjected to a 4-h dark treatment at four different positions in the cycle. The broken lines in these figures show the rhythms in control leaves maintained in continuous light throughout the experiment; they were monitored simultaneously with the rhythms in dark-treated leaves. All the data for a number of such experiments are presented diagrammatically in Fig. 3.56.

In Fig. 3.52 leaves were exposed to darkness for 4 h from 0800 h to midday across the first peak in the rhythm. This treatment resulted in a clear phase shift. Net CO_2 output was observed for about 8 - 10 h after returning the leaves to continuous light and the first post-treatment peak occurred approximately 24 h after the end of the dark treatment. Exposing leaves to darkness from 1000 h to 1400 h also shifts the phase of the rhythm (Fig. 3.53). Emission of CO_2 occurs for approximately 8 - 10 h after the end of the dark treatment and a period of approximately 24 h again elapsed between the end of this treatment and the time of occurrence of the first peak. A phase shift is also induced on exposing leaves to darkness from midday to 1600 h (Fig. 3.54). When the treatment ends at this time, however, there is no 8-h period after the end of the treatment during which the leaves give out CO_2; the rate of CO_2 output decreased rapidly on returning the leaves to LL and the first peak occurred approximately 10 h after the end of the treatment. The slight difference in the lengths of the period in the two samples of treated leaves accounts for the phase difference between the two rhythms. In Fig. 3.55 leaves were exposed to darkness from 1700 h to 2100 h in a trough in the rhythm. This treatment was ineffective in inducing a phase shift. During the dark treatment the leaves continued to fix CO_2 in a similar manner to the control leaves. The next peak of the rhythm in treated leaves occurred about 9 h after the end of the dark treatment and coincided with the corresponding peak in the control leaves.

FIGURE 3.52. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination and normal air at 15°C by exposure to darkness for 4 h from 0800 h to midday as indicated by the arrows. The rhythms in two samples of treated leaves are represented by the continuous lines and those in untreated control leaves by the broken line. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in μg CO_2 h^{-1} g (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.52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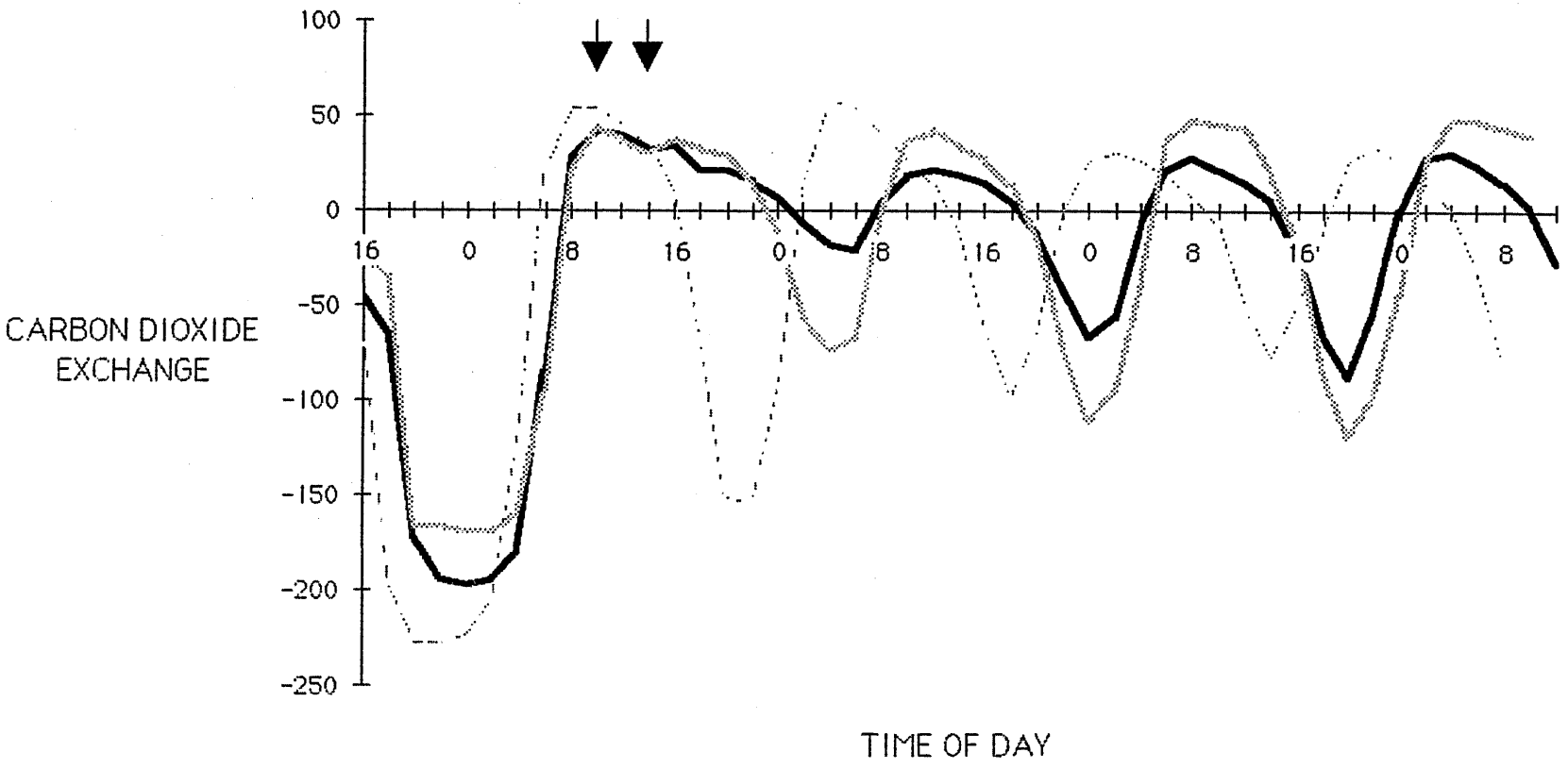
-50
-100
-150
-200
16
0
8
16
0
8
16
0
8
16
0
8
18
2
10
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.53. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination and normal air at 15°C by exposure to darkness for 4 h from 1000 h to 1400 h as indicated by the arrows (continuous line). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.52.

FIGURE 3.53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0
-50
-100
-150
-200
-250
16
0
8
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.54. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination and normal air at 15°C by exposure to darkness for 4 h from midday to 1600 h as indicated by the arrows (continuous lines). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.52.

FIGURE 3.54

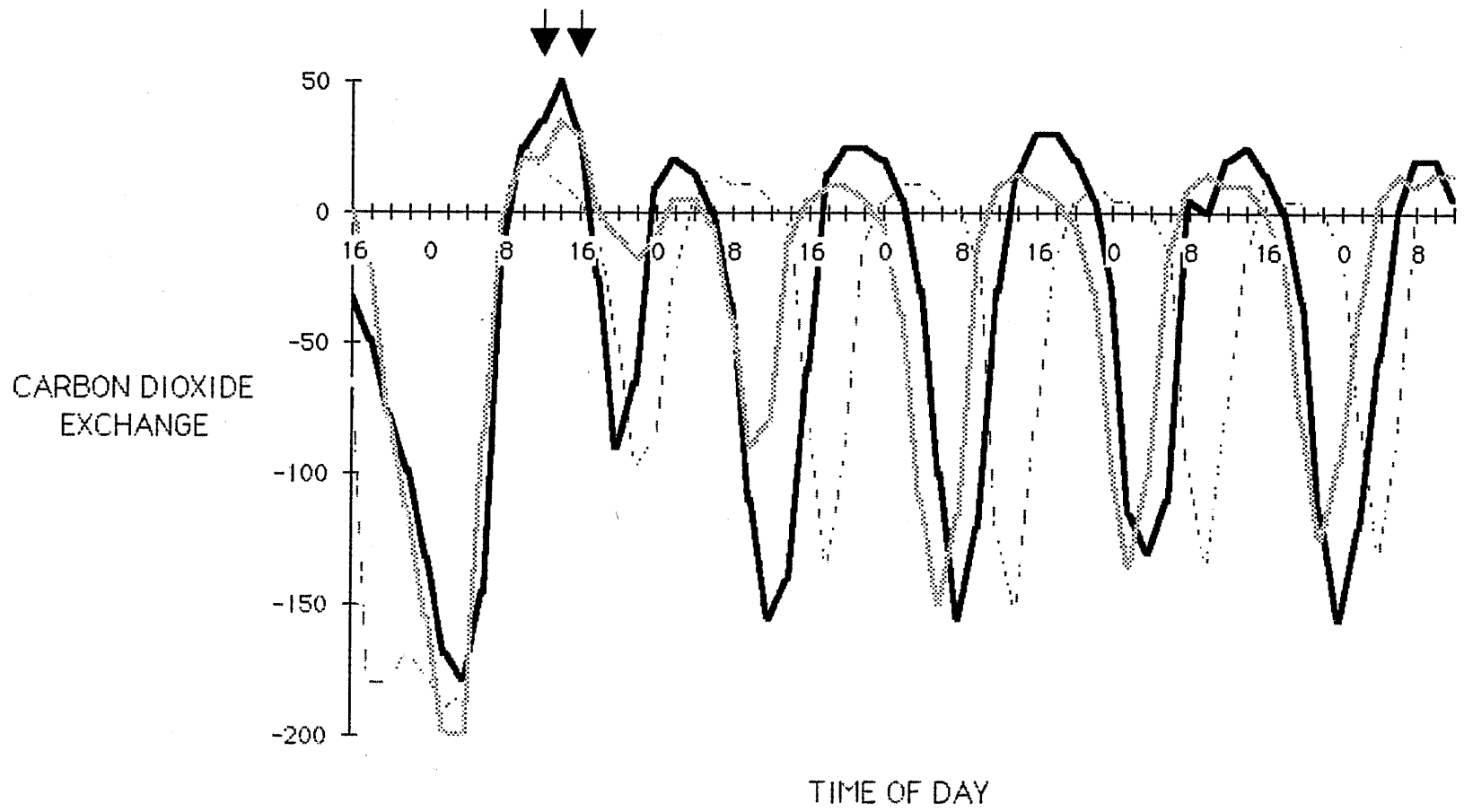

50
0
-50
-100
-150
-200
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.55. The effect of exposing two samples of leaves of *Bryophyllum fedtschenkoi* maintained in continuous light and normal air at 15°C to darkness for 4 h from 1700 h to 2100 h as indicated by the arrows (continuous lines). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.52.

FIGURE 3.55

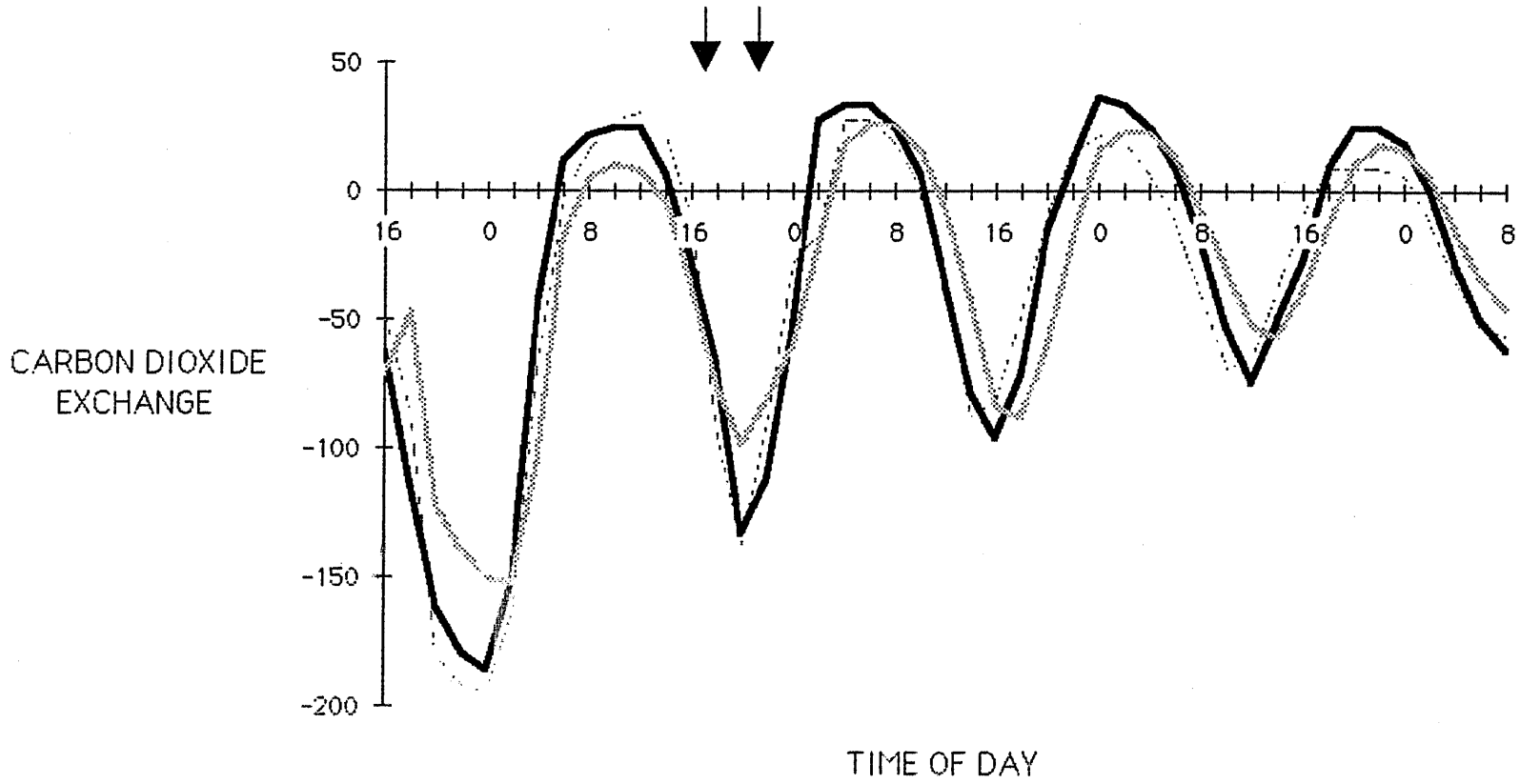
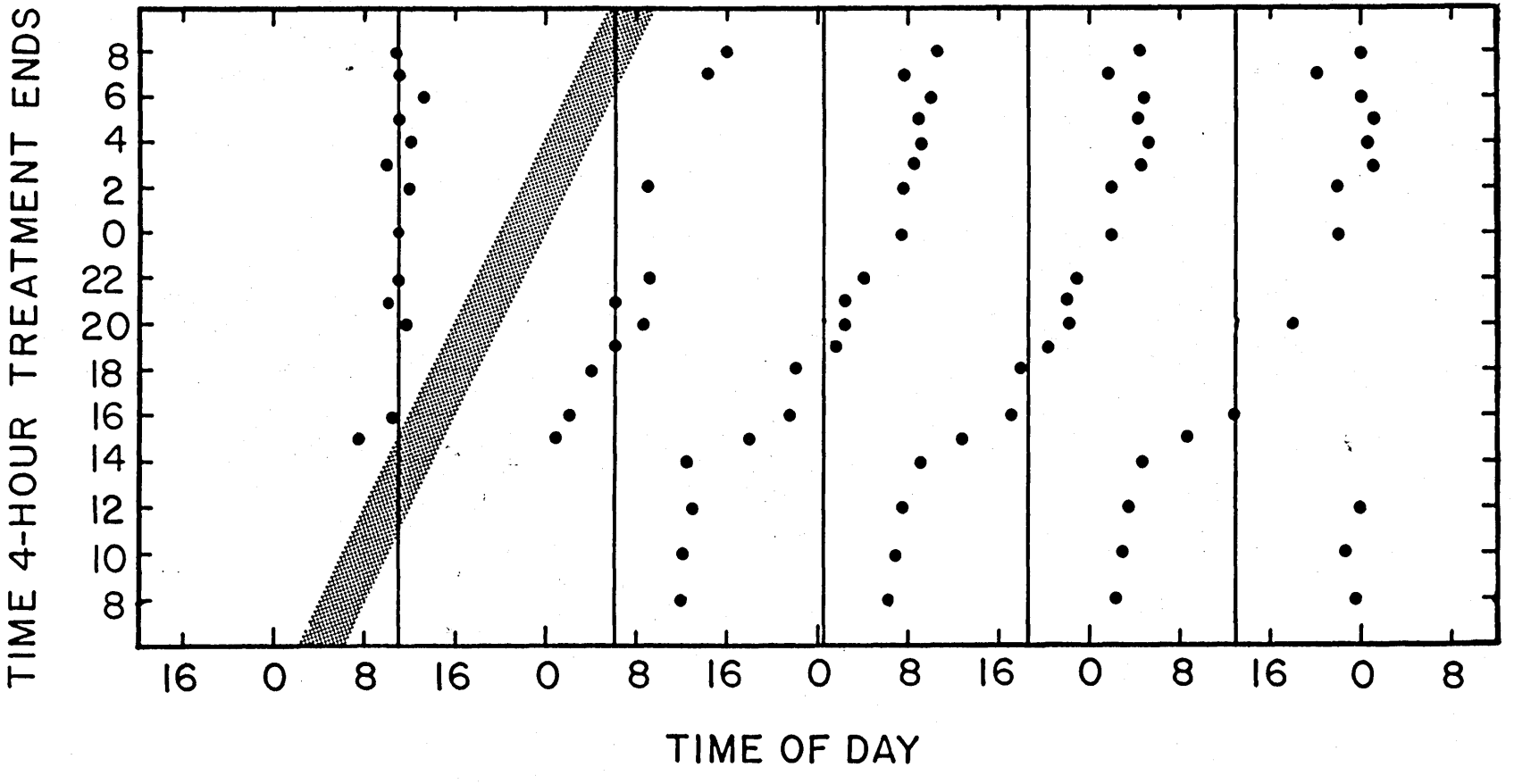

50
0
-50
-100
-150
-200
16
0
8
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.56. Collated data for a series of experiments in which the cycle of CO_2 exchange in *Bryophyllum* leaves was scanned at approximately hourly intervals with a 4-h treatment during which time the leaves were placed in darkness. Leaves were otherwise maintained in continuous light and normal air at 15°C. Times of occurrence of the peaks of untreated control rhythms are represented by the vertical lines. Points in any horizontal line show the mean times of occurrence of the peaks of rhythms in at least two samples of leaves following a treatment at the time indicated on the ordinate. Treatment times are indicated by the shaded bar.


TIME 4-HOUR TREATMENT ENDS
8
6
4
2
0
22
20
18
16
14
12
10
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
TIME OF DAY

The results presented here appear to indicate that the magnitude of the phase shift induced by exposing leaves to darkness for 4 h is not simply related to the position in the cycle at which the dark treatment is applied. There appears to be no fixed relationship between the time in the cycle at which the dark treatment ends and the occurrence of the next peak in the rhythm. This view is supported by the collated data presented in Fig. 3.56. Since the first peak following a dark treatment is not always detectable and, the magnitude of the phase shift on successive peaks is not stable, a point which shall be discussed more fully later, when values for phase shifts are discussed they refer to the phase shift induced on the second peak after the dark treatment.

Although the data in Fig. 3.56 illustrate the existence of a rather complex relationship between the position in the cycle at which a dark treatment is applied and the magnitude of the induced phase shift, a few general trends are nevertheless obvious. Phase shifts induced by treatments ending between 0800 h and 1400 h, that is from just before the first peak until a few hours after the peak, appear to be proportional to the duration of the treatment. A 4-h dark treatment ending between these positions in the cycle delays the phase of the next detectable peak by approximately 6 h. A somewhat similar phase shifting pattern occurs in response to treatments ending between 2200 h and 0800 h on the third day of the experiment, in other words from just after a trough in the rhythm to just after the second peak. At these positions in the cycle a 4-h dark treatment shifts the phase of the rhythm by approximately 8 h. Treatments ending between approximately 1500 h and 2200 h induce relatively small phase shifts, particularly those ending in a trough in the rhythm between 1900 and 2100 h. The magnitude of these phase shifts does not appear to be related to the duration of the dark treatment and may depend only on the position in the cycle at which it ends.

In order to establish unequivocally that the magnitude of the phase shift induced by dark treatments is, at some positions in the cycle, related to the duration of the treatment, it would be necessary to subject leaves to dark treatments of

different durations and record the phase shift obtained. Such experiments were not carried out in this study because it was clear that although the relationship between the time in the cycle at which the dark treatment ended and the magnitude of the induced phase shift was more complex than that between the time in the cycle at which a 2°C treatment was applied and the magnitude of the phase shift induced, the positions in the cycle at which the oscillator appeared to be sensitive or insensitive to darkness and to low temperature were nevertheless similar. It was considered to be more important to try to establish whether or not the similarity of the phase shifting effects of darkness and low temperature extended to the direction of the phase shifts induced by these treatments. Since it was proposed to determine the direction of the phase shifts by the same procedure as previously described for high and low temperature, that is by comparing the amount of phase shift induced by a 1-h and 4-h exposure to darkness, it was hoped that these experiments would, in addition, give some indication as to whether or not there was a relationship between the duration of the dark treatment and the magnitude of the phase shift induced.

The dark and light continuous lines in Figs. 3.57 - 3.60 are four examples of the results obtained when leaves were exposed to a dark treatment of either 4 or 1 h duration at four different points in the cycle. In all experiments the 1- and 4-h treatments ended at the same time. The broken lines represent the rhythms in control leaves maintained in continuous light throughout the experiment.

In Fig. 3.57 one sample of leaves was exposed to darkness from 0800 h to midday and the other from 1100 h to midday. The 1-h treatment gave a smaller phase shift than the 4-h treatment. The first peak of the rhythm after exposure of the leaves to darkness for 4 h occurred at midday, approximately 8 h later than the occurrence of the corresponding peak in the untreated control leaves. The first peak following a 1-h treatment occurred at 0800 h, approximately 4 h later than the peak in control leaves. A dark treatment ending at 12 midday therefore delays the phase of the rhythm. Dark treatments ending at 1400 h appear to induce phase advances (Fig.

FIGURE 3.57. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to darkness for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at midday as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (0800 h) and the second arrow the time at which the 1-h treatment began (1100 h). The broken line represents the rhythm in untreated control leaves. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g$ (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.57

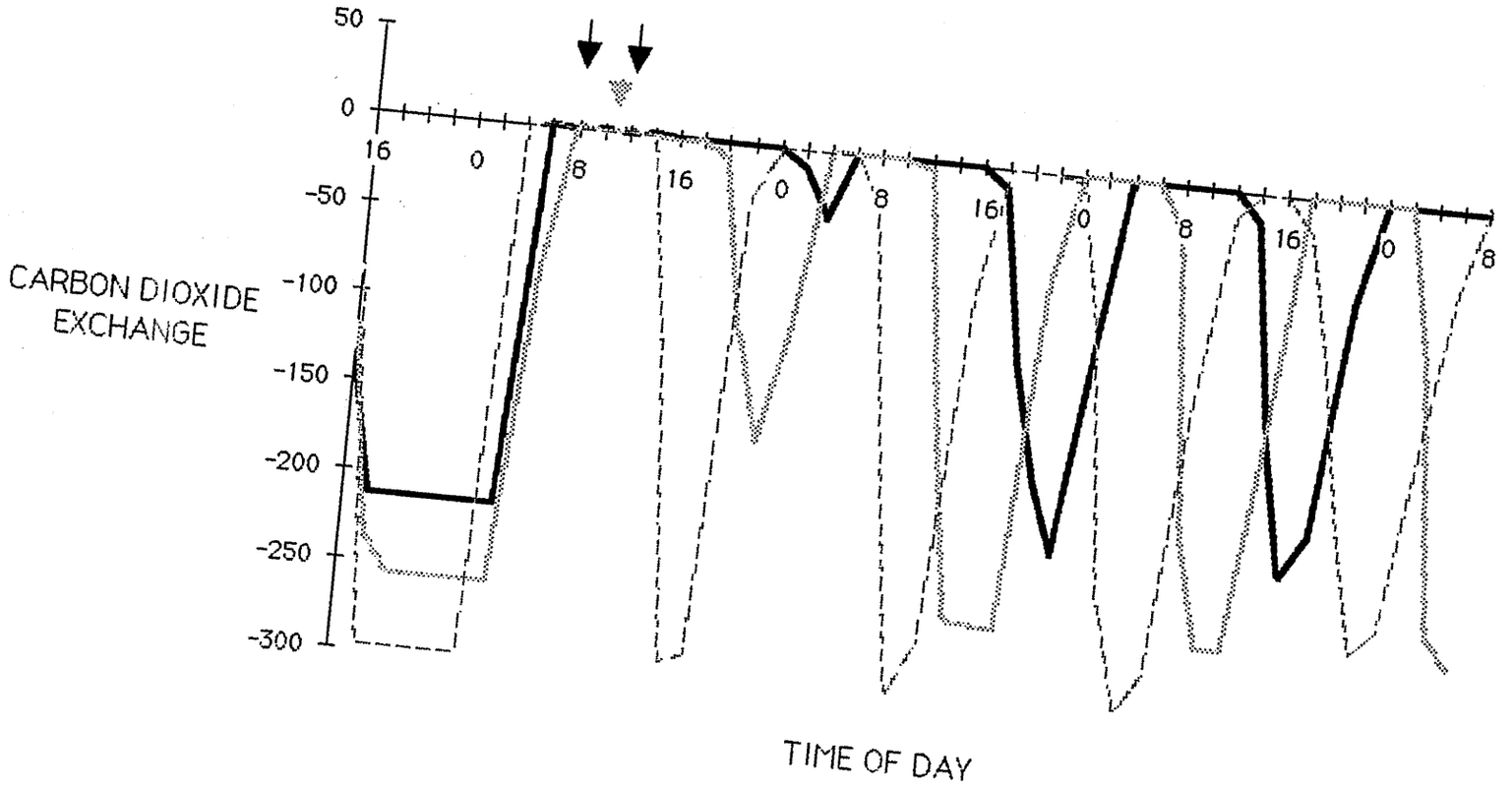
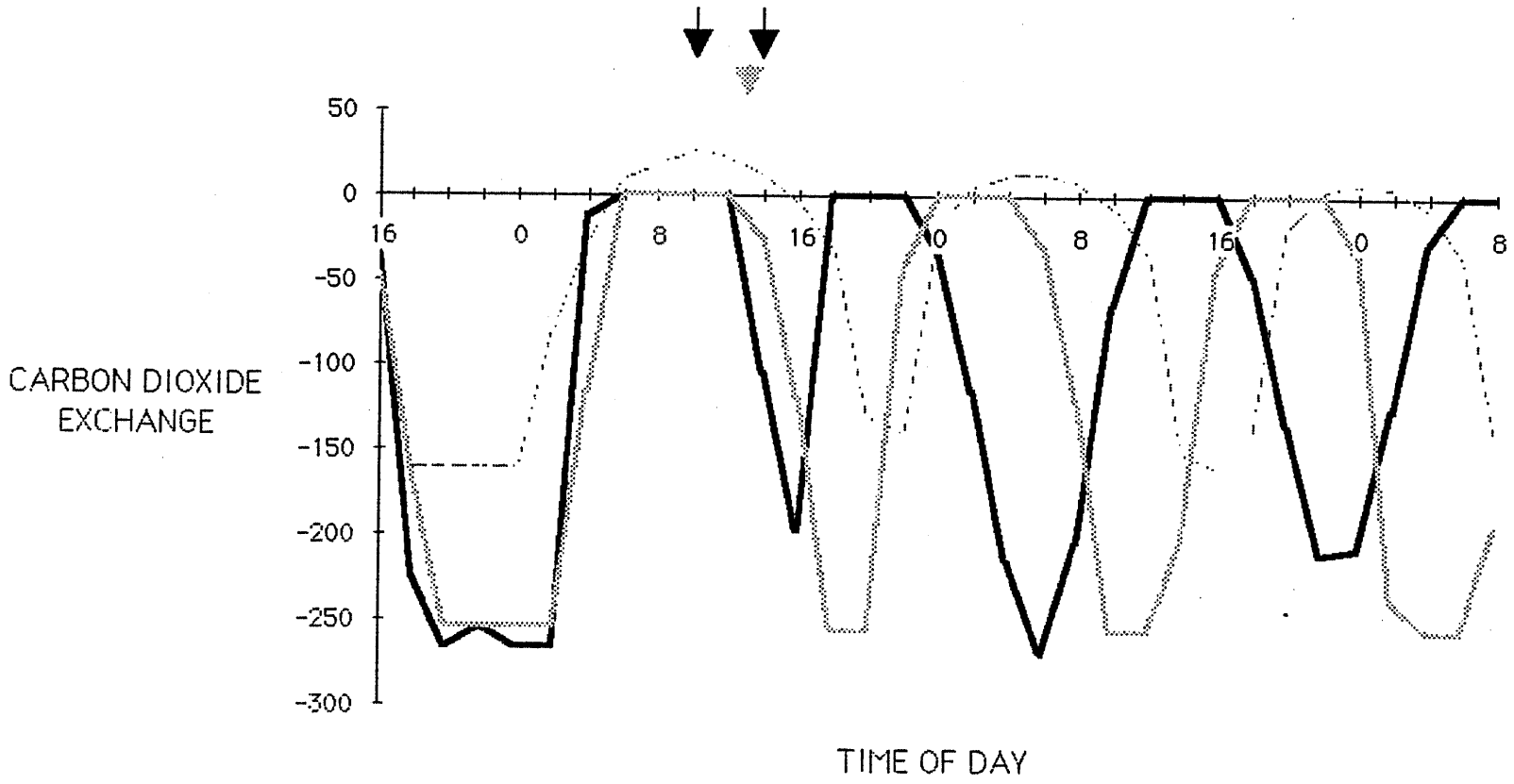

50
0
-50
-100
-150
-200
-250
-300
CARBON DIOXIDE EXCHANGE
16
0
8
16
0
8
16
0
8
16
0
8
TIME OF DAY

FIGURE 3.58. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to darkness for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at 1400 h as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (1000 h) and the second arrow the time at which the 1-h treatment began (1300 h). The broken line represents the rhythm in untreated control leaves. Presentation as for Fig. 3.57.

FIGURE 3.58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-100
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-200
-250
-300
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.59. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to darkness for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at 1600 h as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (midday) and the second arrow the time at which the 1-h treatment began (1500 h). The broken line represents the rhythm in untreated control leaves. Presentation as for Fig. 3.57.

FIGURE 3.59

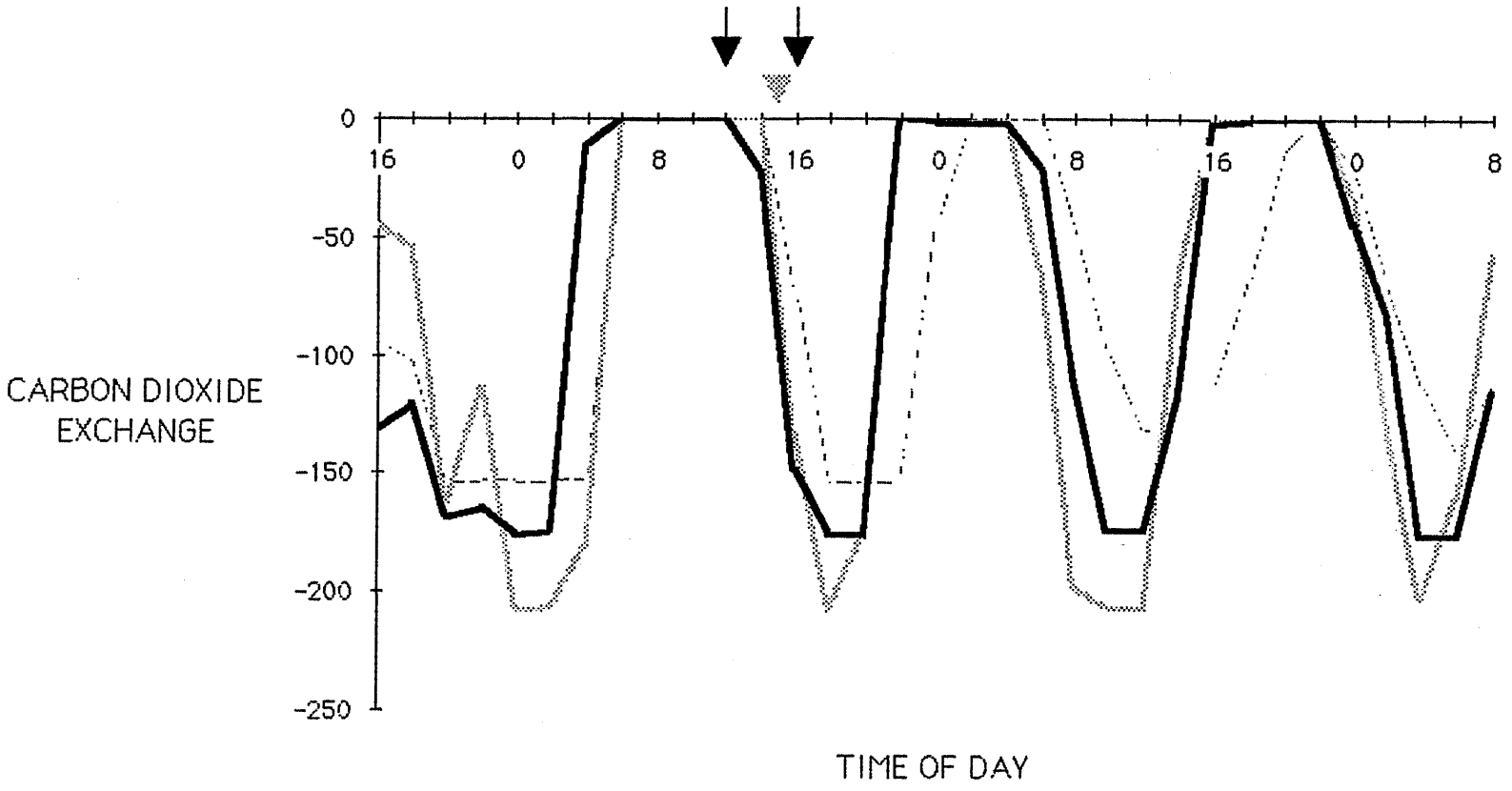

0
-50
-100
-150
-200
-250
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.60

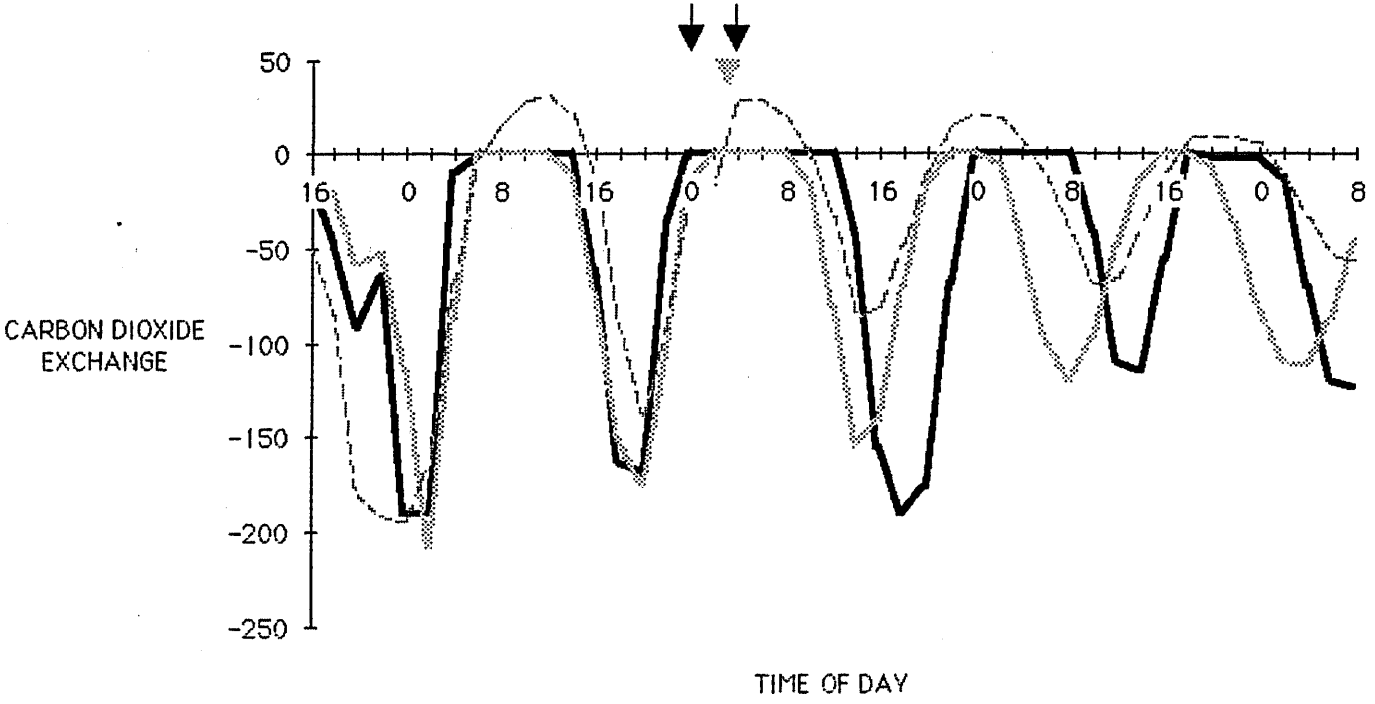
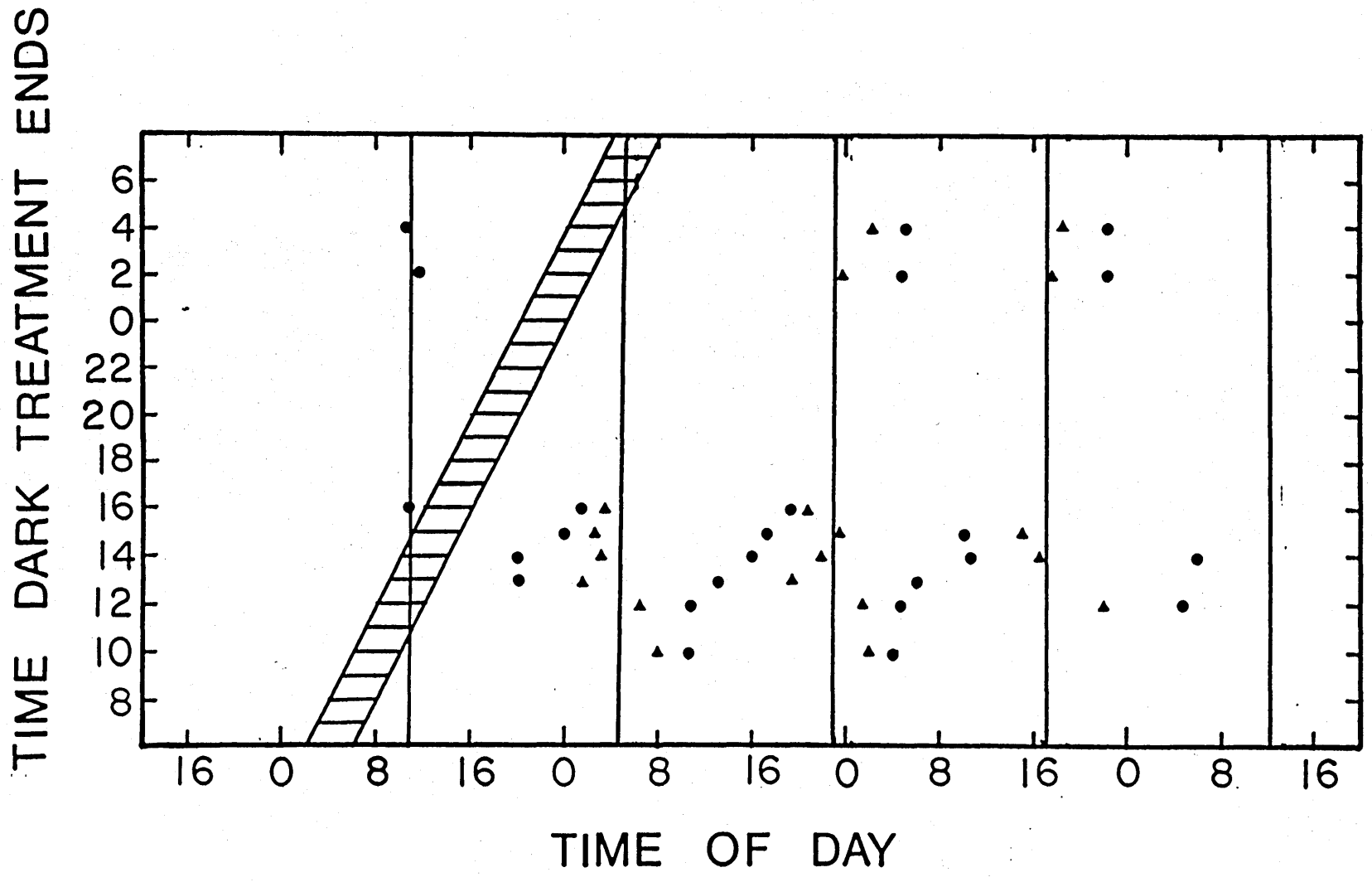

50
0
-50
-100
-150
-200
-250
16
0
8
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.60. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and normal air at 15°C by exposure to darkness for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at 0400 h as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (midnight) and the second arrow the time at which the 1-h treatment began (0300 h). The broken line represents the rhythm in untreated control leaves. Presentation as for Fig. 3.57.

FIGURE 3.61. Collated data for a series of experiments in which the cycle of CO_2 exchange in *Bryophyllum* leaves was scanned with a 4-h (●) or 1-h (▲) treatment during which time the leaves were exposed to darkness. Leaves were otherwise maintained in continuous light and normal air at 15°C. Times of occurrence of the peaks of untreated control rhythms are represented by the vertical lines. Points in any horizontal line show the mean times of the occurrence of the peaks of the rhythms in at least two samples of leaves following a treatment at the time indicated on the ordinate. Treatment times are indicated by the shaded bar.


TIME DARK TREATMENT ENDS
6
4
2
0
22
20
18
16
14
12
10
8
16
0
8
16
0
8
16
0
8
16
0
8
16
TIME OF DAY

3.58). The 4-h treatment advanced the phase of the next peak by approximately 8 h whilst the 1-h treatment induced an advance of approximately 3 h. A phase advance is also induced by a dark treatment ending at 1600 h. The results of this experiment are shown in Fig. 3.59. These curves appear to indicate that the 1- and 4-h treatments induce phase shifts of equal magnitude. However, it is clear from the collated data presented in Fig. 3.61, where the averages of two experiments have been plotted, that the phase advance induced by a 4-h treatment is slightly greater than that produced by a 1-h treatment. A 4-h exposure to darkness at this position in the cycle appears to advance the phase of the following peak by approximately 4 h while a 1-h treatment induces a phase advance of approximately 2 h. A dark treatment ending at 0400 h on the third day of the experiment appears to delay the phase of the rhythm as shown in Fig 3.60. The first peak following a 4-h treatment is delayed by approximately 4 h but scarcely any phase shift is induced by the 1-h treatment. In fact the first peak following the 1-h dark exposure appears to occur about 1 h earlier than the corresponding control peak. Nevertheless, the collated data in Fig. 3.61 indicate that a dark treatment ending at 0400 h on the third day of the experiment delays the phase of the rhythm.

The collated data (Fig. 3.61) indicate that dark treatments ending between 1000 h and midday on the second day of the experiment, that is, across a peak in the rhythm, induce phase delays. Treatments ending a few hours after a peak until a few hours before a trough, from 1300 h to 1600 h, advance the phase of the rhythm. These results are closely similar to those found for low temperature. The cross-over point between phase advances and phase delays occurs at 1300 h, the same time at which the cross-over occurred with low temperatures.

The results presented in Fig. 3.56 suggested that between 0800 h and 1400 h the magnitude of the phase shift induced by a dark treatment is related to the duration of the treatment. The results in Fig. 3.61, however, indicate that this relationship does not extend to treatments ending at 1400 h since at 1300 h phase advances are induced while between 0800h and midday phase delays occur. The results in Fig. 3.61

do, however, provide some evidence to support the view that phase shifts induced by treatments ending between 1000 h and midday are related to the duration of the treatment. A 4-h treatment ending between these times induced a phase shift of about 5 h while a 1-h treatment delayed the phase by about 1 - 2 h.

The average length of the period in leaves subjected to darkness for 4 h was 19.4 ± 0.21 h, a value significantly longer than the period of 18.4 ± 0.19 h recorded in control leaves monitored simultaneously with the treated leaves. This result, which has been calculated using the data obtained from experiments carried out to determine the magnitude of the phase shift, (Figs. 3.52 - 3.56) is somewhat surprising, particularly in view of the finding that after prolonged exposures to darkness the period of the rhythm initiated on transferring leaves to LL was shorter than that in control leaves. The reason for this discrepancy is uncertain but it is possible that a temporary instability in the period may occur after a dark treatment. The difference in the lengths of the period in dark-treated and control leaves accounts for the fact that the phase shift induced by dark treatments is not stable but becomes either greater or smaller on successive peaks depending upon whether the phase is advanced or delayed as seen in Fig. 3.56.

The period of the rhythms recorded during experiments to determine the direction of the phase shift are somewhat shorter than expected. The periods of these rhythms are given in Table 8.

TABLE 8

COMPARISON OF PERIODS IN CONTROL AND DARK-TREATED LEAVES

TREATMENT	PERIOD LENGTH
continuous light	17.6 ± 0.52 h
4 h dark	17.7 ± 0.41 h
1 h dark	17.9 ± 0.33 h

The reason for the apparently shorter period of the control rhythm recorded during these experiments is not known. Only a limited number of periods were

available from these experiments to determine the lengths of the period. It is perhaps significant that just prior to carrying out this series of experiments the fluorescent tube above the leaf chambers was replaced and the light intensity provided by the new tube, approximately 17 μmol m^{-2} s^{-1} was 5 - 6 μmol m^{-2} s^{-1} greater than that provided by the old tube. A similar shortening of the period was observed in experiments carried out in another set of apparatus after replacing the fluorescent tube. These results are discussed in the next section. The important point to note here, however, is that no significant differences were found between the period of the untreated control leaves and the period in leaves which had been subjected to darkness for either 1 or 4 h. These results do not therefore confirm the previous finding that the period becomes longer after a 4-h exposure to darkness.

Since leaves appear to accumulate malate in continuous darkness it is possible that a 4-h dark treatment exerts its effect on the phase by altering the malate status of the leaves. An attempt has therefore been made to test this possibility.

Twelve leaves were placed in continuous illumination at 15°C at the end of the normal growth-room photoperiod. The following day six of these leaves were exposed to darkness from 1000 h to 1400 h while the other six remained in continuous illumination. The reason for exposing leaves to darkness at this time was that a large phase shift had previously been found to occur in response to a dark treatment applied in this position in the cycle. Immediately after the end of the dark treatment at 1400 h, samples of treated and untreated leaves were removed from the leaf chambers and the cell sap extracted. The malate content of the dark-treated and untreated control leaves was then compared. The results of two independent experiments (1st two rows) and the average results of both (3rd row) shown in Table 9.

TABLE 9

COMPARISON OF THE MALATE CONTENT IN LEAVES MAINTAINED IN CONTINUOUS ILLUMINATION AND THOSE EXPOSED TO DARKNESS FOR 4 h

EXP. NO.	mM MALATE IN CELL SAP			
	CONTROL	DARK TREATED	t	S
1	30.22 ± 2.2	27.28 ± 1.7	1.05	N.S.
2	26.89 ± 3.1	25.57 ± 1.4	0.62	N.S.
1+2	29 .06 ± 1.7	26.48 ± 1.2	0.202	N.S

These results reveal that no significant change in the malate concentration in the extraced cell sap occurs in response to a 4-h dark treatment ending at 1400 h on the second day of the experiment. It seems unlikely that dark treatments exert their effect on the phase by altering the gross malate status of the leaves. The rhythms in leaves used in these experiments were monitored up to the time they were extracted to ensure that the leaves were at the phase point where a dark treatment would be expected to induce a large phase shift in the rhythm and, with the exception of one sample of leaves, this was found to be the case.

The most important findings to emerge from this section of the investigation are therefore as follows:

1. Prolonged exposure of the leaves to darkness inhibits the rhythm of CO_2 exchange apparently by forcing the basic oscillator to, and holding it at, a fixed phase point in the cycle characterised by the leaf cells containing relatively large amounts of malate.

2. Exposing leaves to darkness for a few hours shifts the phase of the rhythm. The magnitude of the phase shift depends upon the position in the cycle at which the dark treatment is applied. At several positions in the cycle, however, a dark

treatment induces a phase shift which appears to be related to the duration of the treatment.

4. The oscillator is sensitive to dark treatments in those positions in the cycle at which it is also sensitive to low temperature. The similarity of the phase shifts induced by those treatments extends to the direction of the phase shift induced.

5. The phase shifts induced by short dark treatments are not accompanied by a detectable change in the malate concentration in the sap expressed from the leaf cells.

3.3. THE EFFECTS OF 5% CO_2 ON THE RHYTHM OF CO_2 EXCHANGE

The results presented in the previous two sections have indicated that prolonged exposures to low temperature and darkness inhibit the rhythm of CO_2 exchange exhibited by leaves of *Bryophyllum fedtschenkoi* in a similar manner, apparently by preventing malate breakdown thereby leading to the leaves becoming relatively rich in malate. The apparent differences in the detailed characteristics of the phase shifts induced by short exposures to low temperature and darkness suggest that the precise way in which malate breakdown is prevented by these two treatments may differ. If darkness inhibits the rhythm because the enzyme responsible for malate breakdown is inhibited by the end products, then a similar inhibition would be expected to occur in response to increased ambient CO_2 concentrations. In this section a detailed study of the effects of increasing the CO_2 concentration on the rhythm has been made with the aim of establishing the following points:

1. Whether or not the rhythm is inhibited under high concentrations of CO_2, and if so the nature of the inhibition.

2. The malate status of leaves maintained in high CO_2 concentrations.

3. Whether or not short exposures to 5% CO_2 induce phase shifts in the rhythm and, if so, whether or not the characteristics of the phase shifts are similar to those induced by dark treatments.

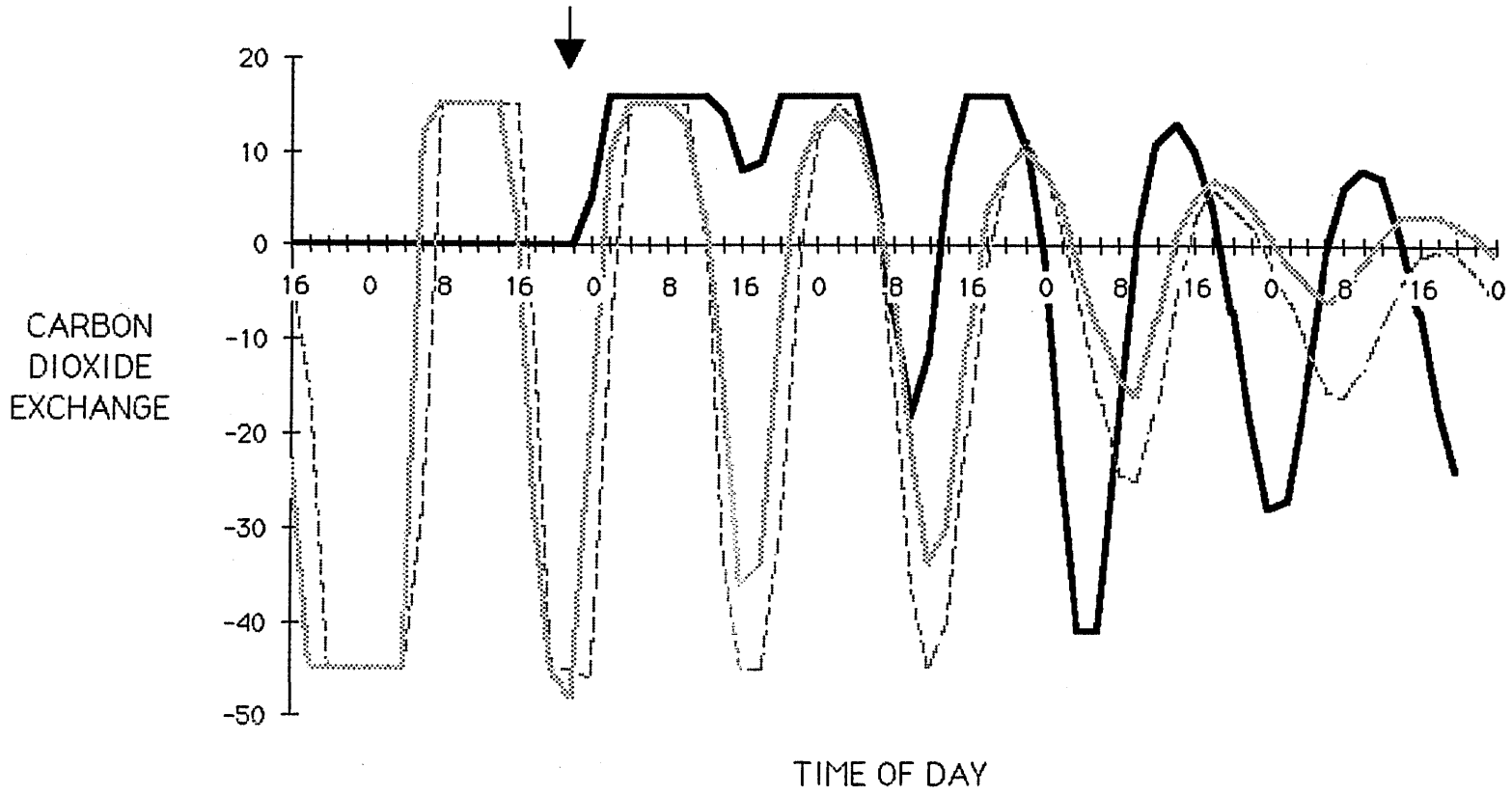
3.3.1. THE EFFECTS OF PROLONGED EXPOSURES TO 5% CO_2

The continuous lines in Figs. 3.62 and 3.63 show the results of exposing leaves to 5% CO_2 for a few days and then transferring them to a stream of normal air. The broken lines represent the rhythms in two samples of control leaves maintained throughout the experiment in a stream of normal air. Both treated and control leaves were otherwise kept in continuous illumination at 15°C. During the 5% CO_2 treatment the pattern of CO_2 exchange in the leaves was not monitored because the IRGA could not detect changes in CO_2 concentration at high ambient levels. The time at which leaves were transferred from 5% CO_2 to normal air was 2200 h on the second day of the experiment in Fig. 3. 62, and 1000 h on the fourth day of the experiment in Fig. 3. 63.

Following the transfer from 5% CO_2 to normal air there was a period of between 4 and 14 h during which CO_2 was given out at such a high rate that the IRGA was forced off the scale. After this time the rate of CO_2 output began to decrease, the first peak of the rhythm occurred approximately 23 h after the end of the treatment regardless of the time of day at which the leaves were placed in normal air. This point is illustrated by the collated data presented in Table 10 where the times taken to reach the peaks after a 5% CO_2 treatment are the mean times taken by at least two samples of leaves.

FIGURE 3.62. The rhythm of CO_2 exchange exhibited by leaves of *Bryophyllum fedtschenkoi* transferred from 5% CO_2 to normal air at 2200 h as indicated by the arrow (dark continuous line). Leaves were otherwise maintained in continuous illumination at 15°C. The light continuous line and broken line represent the rhythms in two samples of control leaves maintained continuously in normal air. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.62.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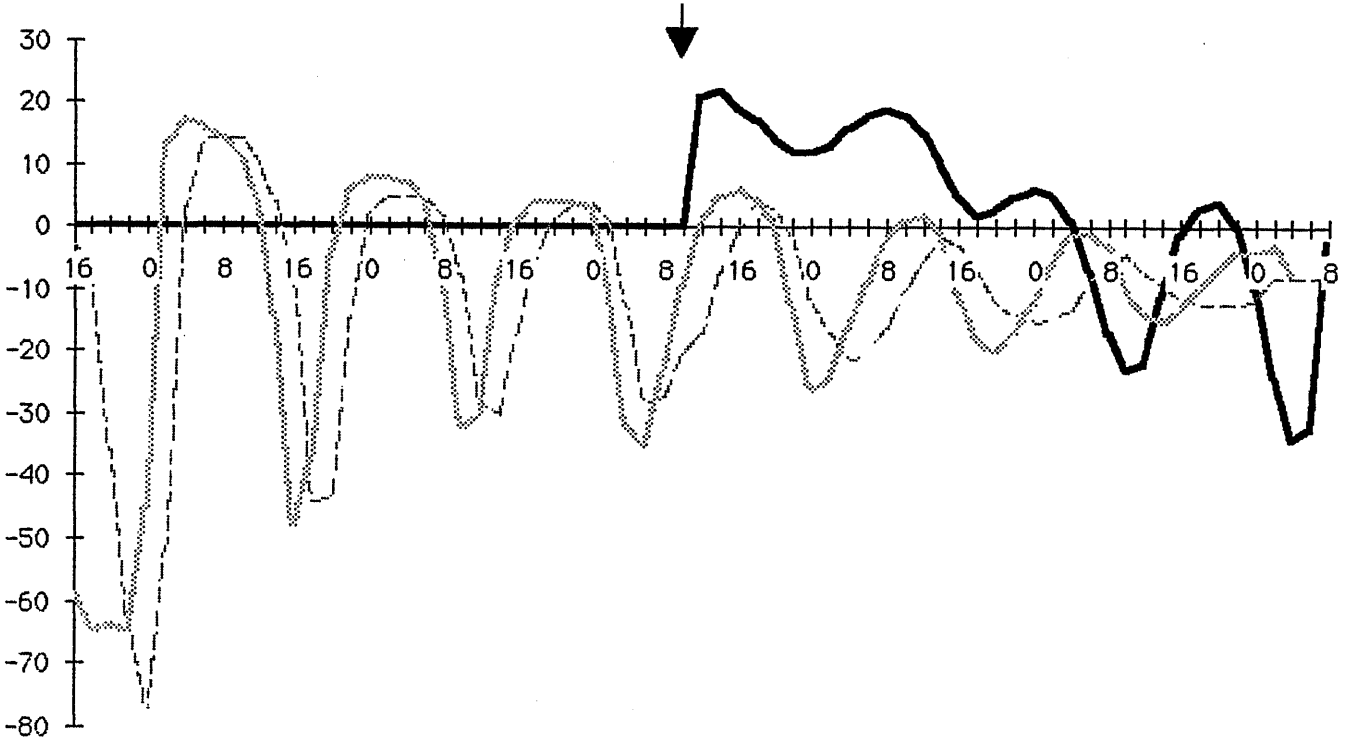
-20
-30
-40
-50
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
CARBON
DIOXIDE
EXCHANGE
TIME OF DAY

FIGURE 3.63. The rhythm of CO_2 exchange exhibited by leaves of *Bryophyllum fedtschenkoi* transferred from 5% CO_2 to normal air at 1000 h as indicated by the arrow (dark continuous line). Leaves were otherwise maintained in continuous illumination at 15°C. The light continuous line and broken line represent the rhythms in two samples of control leaves maintained continuously in normal air. Presentation as for Fig. 3.62.

FIGURE 3.63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10
0
-10
-20
-30
-40
-50
-60
-70
-80
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

TABLE 10

RELATIONSHIP BETWEEN END OF 5% CO_2 TREATMENT AND OCCURRENCE OF PEAKS

TIME(h)	TIME (h) TO:		
IN 5% CO_2	1st Peak	2nd Peak	3rd Peak
20	23	40	60
32	24	44	63
48	23	42	61
66	22	38	57

Since the pattern of CO_2 exchange could not be monitored during the 5% CO_2 treatment no record of the possible occurrence of rhythmicity could be obtained during the time at which the leaves were exposed to this treatment. But the fact that the first peak of the rhythm always occurred a definite time after the end of the 5% CO_2 treatment strongly indicates that during exposure to high concentrations of CO_2 the rhythm is in fact, inhibited, probably by the oscillating system being held at a fixed phase point in the cycle. The 4 - 14 h after the end of the treatment during which CO_2 is given out at a high rate by the leaves may reflect a period in which the leaf cells are decarboxylating malate. Moreover, the time which elapsed between the end of the 5% CO_2 treatment and the occurrence of the first peak, about 23 h, is similar to the time taken to reach the first peak after a dark treatment (24 h). Thus 5% CO_2 appears to inhibit the rhythm in a similar manner to darkness.

The rhythm which is detected in leaves following transfer from 5% CO_2 to normal air continued for at least 4 days, and the first cycle often reflected only a rhythm in CO_2 output. The 5% CO_2 treatment apparently has no effect on the subsequent period of oscillation in normal air. The period in leaves subjected to a 5 %

CO_2 treatment, 19.2 ± 0.61 h is not significantly different from the period of 18.8 ± 0.32 h recorded in control leaves maintained in normal air throughout the experiment. In this respect therefore, the effects of low temperature and darkness appear to differ from those of 5% CO_2.

If exposing leaves to 5% CO_2 inhibits the rhythm in a way similar to that in which darkness and low temperature inhibit oscillation, then leaves would be expected to accumulate relatively large amounts of malate during prolonged exposure to 5% CO_2. This possibility was tested by measuring the malate status of leaves maintained in 5% CO_2 for various lengths of time and the results of this study are presented in Fig. 3.64.

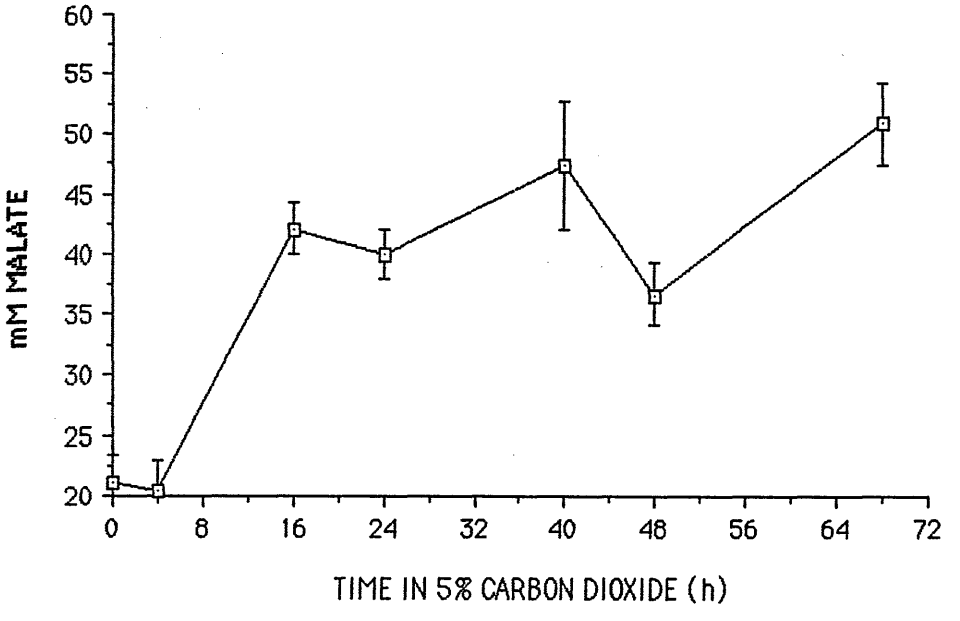


Figure 3.64. Concentration of malate in the extracted cell sap of leaves maintained in 5% CO_2 and continuous illumination at 15°C. Each point on the curve is the average concentration of malate in the sap of 6 leaves. The vertical lines represent ± the standard errors of the means. Ordinate: concentration of malate in mM. Abscissa: Time in 5% CO_2 (h).

Very little change in the malate concentration of the extracted cell sap occurs during the first 4 h in 5% CO_2. After this time the concentration of malate increases from approximately 20 mM to 40 mM 16 h after the beginning of the experiment and thereafter remains relatively constant. These results are therefore consistent with the view that prolonged exposures to 5% CO_2 inhibit oscillation by causing the leaf to acquire relatively high concentrations of malate, and in this respect the effects of exposing leaves to high concentrations of CO_2 are indeed similar to the effects of exposing leaves to darkness and low temperature. It was therefore interesting to establish whether or not short exposures to 5% CO_2 were effective in inducing phase shifts in the rhythm of CO_2 exchange, and, if so, whether or not the oscillator was sensitive to 5% CO_2 in similar positions in the cycle at which it had previously been found to be sensitive to low temperature and darkness.

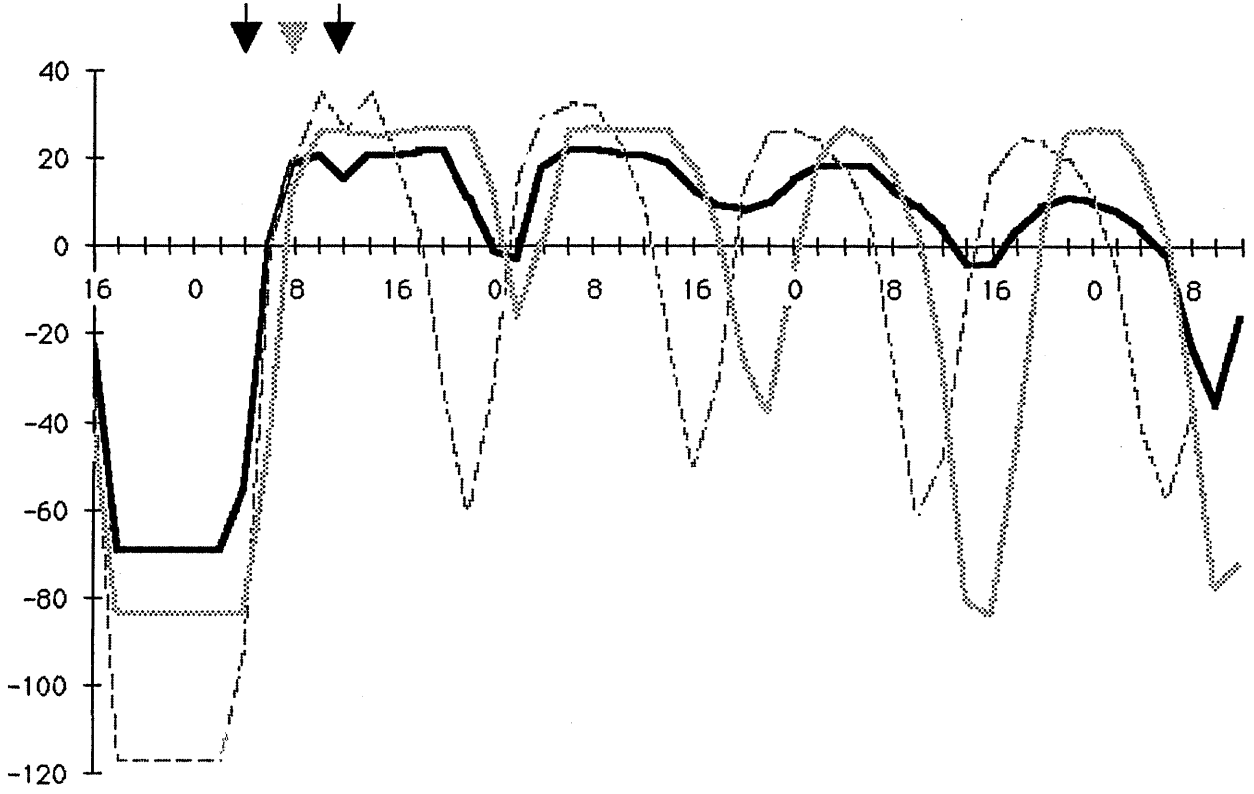
3.3.2. THE EFFECTS OF SHORT PULSE-TYPE 5% CO_2 TREATMENTS ON THE RHYTHM OF CO_2 EXCHANGE

The phase points in the circadian cycle at which the oscillator is sensitive to high concentrations of CO_2 have been established by subjecting leaves to 5% CO_2 for 4 h at various positions in the cycle.

Examples of the curves obtained when leaves were exposed to 5% CO_2 for 4 h at four different positions in the cycle are shown by the continuous lines in Figs. 3.65 and 3.66. The broken lines indicate the rhythm in control leaves maintained continuously in a stream of normal air. As in previous experiments, CO_2 exchange

FIGURE 3.65. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination and normal air at 15°C by exposure to 5% CO_2 for 4 h from either 0400h to 0800 h (dark continuous line) or from 0800 h to midday (light continuous line) as indicated by the arrows. The rhythm in untreated control leaves is represented by the broken line. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in μg CO_2 h^{-1} g (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.65


40
20
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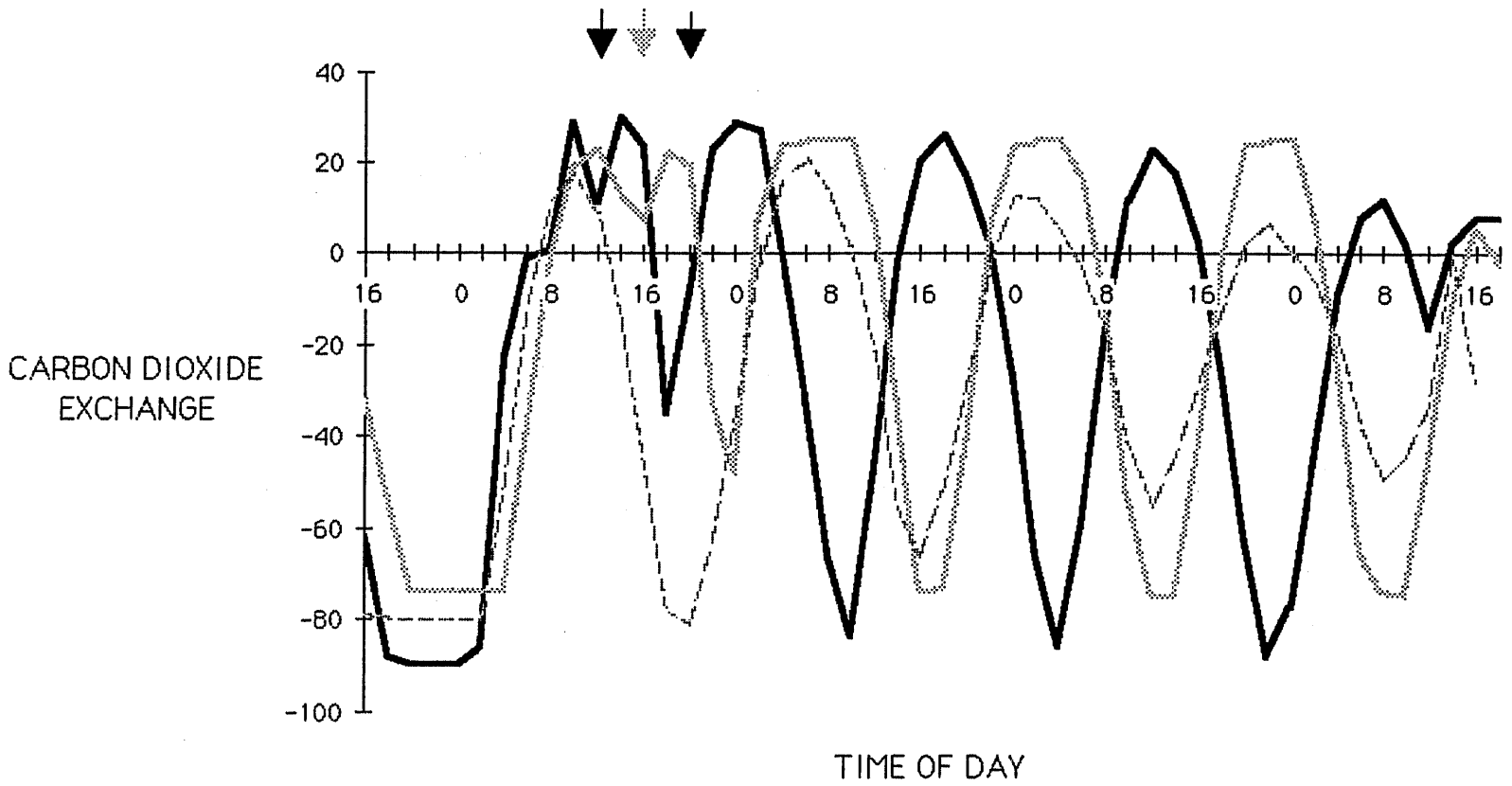
-20
-40
-60
-80
-100
-120
16
0
8
16
0
8
16
0
8
16
0
8
CARBON DIOXIDE EXCHANGE
TIME OF DAY

FIGURE 3.66. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination and normal air at 15°C by exposure to 5% CO_2 for 4 h from either midday to1600 h (dark continuous line) or from 1600 h to 2000 h (light continuous line) as indicated by the arrows. The rhythm in untreated control leaves is represented by the broken line. Presentation as for Fig. 3.65.

FIGURE 3.66

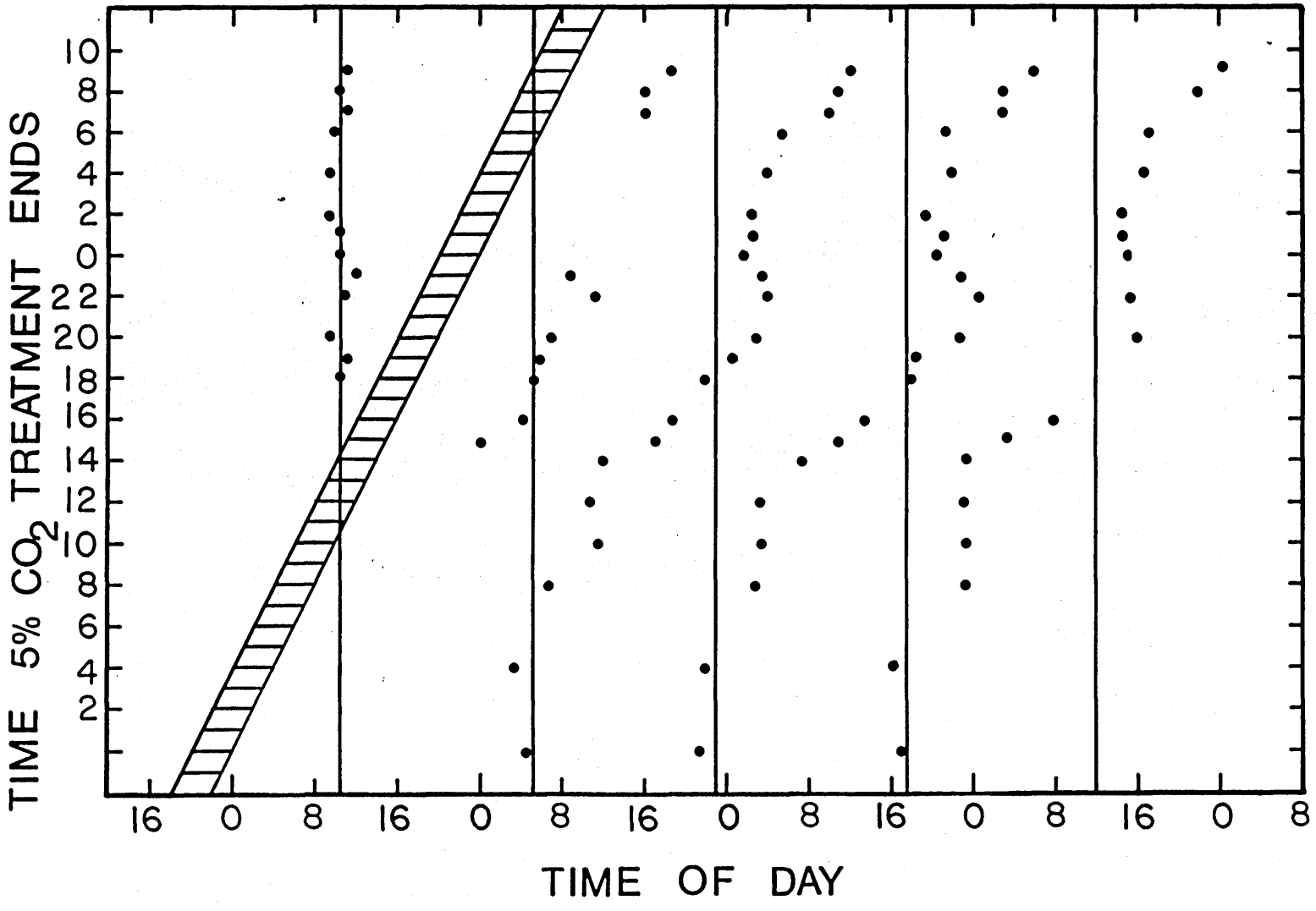

CARBON DIOXIDE EXCHANGE
40
20
0
-20
-40
-60
-80
-100
16
0
8
16
0
8
16
0
8
16
0
8
16
TIME OF DAY

was not monitored during the 5% CO_2 treatment, but the values of CO_2 uptake or output recorded immediately prior to and after the end of the treatment have been joined.

In Fig. 3.65 the darker of the two continuous lines shows the effect of exposing leaves to 5% CO_2 from 0400 h to 0800 h and the lighter line from 0800 h to midday. After transferring leaves from 5% CO_2 to normal air there is a period of 10 - 12 h during which CO_2 is given out by the leaves at a very high rate, thereafter the rate of CO_2 output begins to decrease. When the 5% CO_2 treatment ended at 0800 h the first peak of the rhythm occurred approximately 26 h later, whereas when it ended at midday, the first peak of the rhythm occurred 22 h later. Thus, treatments ending at 0800 h and midday result in phase shifts of nearly equal magnitude, the peaks in the treated leaves apparently occurring about 3 - 4 h later than the corresponding peaks of the rhythm in control leaves. Exposing leaves to 5% CO_2 from midday to 1600 h, as shown by the dark continuous line in Fig. 3.66, also induces a phase shift. However, CO_2 is not given out by the leaves for the first few hours after the end of the treatment. The rate of CO_2 output begins to decrease immediately on transferring leaves to normal air and the first peak of the rhythm occurs approximately 8 h later. When leaves were exposed to 5% CO_2 from 1600 h to 2000 h as shown by the light continuous line in Fig. 3.66, no phase shift was induced. Following the transfer from 5% CO_2 to normal air, the rate of CO_2 output decreased and the next peak occurred approximately 11 h after the end of the treatment coinciding with the corresponding peak in the control rhythm.

The collated data for a number of experiments in which the circadian cycle of CO_2 exchange was scanned with treatments in which the leaves were exposed to 5% CO_2 for 4 h at approximately 2-hourly intervals are presented diagrammatically in Fig. 3.67.

FIGURE 3.67. Collated data for a series of experiments in which the cycle of CO_2 exchange in *Bryophyllum* leaves was scanned at approximately 2-hourly intervals with a 4-h treatment during which time the leaves were exposed to 5% CO_2. Leaves were otherwise maintained in a stream of normal air and continuous light at 15°C. Times of occurrence of the peaks of untreated control rhythms are represented by the vertical lines. Points in any horizontal line show the mean times of occurrence of the peaks of the rhythm in at least two samples of leaves following a treatment at the time indicated on the ordinate. Treatment times are indicated by the shaded bar.


TIME 5% CO_2 TREATMENT ENDS
10
8
6
4
2
0
22
20
18
16
14
12
10
8
6
4
2
16
0
8
16
0
8
16
0
8
16
0
8
16
0
8
TIME OF DAY

These results illustrate the rather complex relationship which exists between the time in the cycle at which a 5% CO_2 treatment ends and the magnitude of the phase shift induced. Although somewhat difficult to interpret, several general trends are obvious. Treatments ending between midnight and 0800 h on the second day of the experiment, induce little or no phase shift. Phase shifts induced by treatments ending between the approximate times of 0800 h and 1400 h appear to be related to the duration of the treatment. Exposing leaves to 5% CO_2 for 4 h between these times shifts the phase of the following peaks by approximately 4 - 6 h. A similar result is observed for treatments ending between the approximate time limits of 2000 h and 0600 h. Treatments ending between these positions in the cycle also induce phase shifts of about 4 - 6 h. When the 5% CO_2 treatment ends between approximately 1500 h and 1900 h relatively small phase shifts are induced which do not appear to bear a relationship to the duration of the treatment but may be related only to the time in the cycle at which the treatment ends.

The phase shifting pattern induced by scanning the cycle of CO_2 exchange with 5% CO_2 treatments is remarkably similar to that produced by short dark treatments. The positions in the cycle at which 5% CO_2 treatments induce phase shifts which appear to be related to the duration of the treatment are similar to those at which dark treatments induced phase shifts which were related to the duration of the treatment. Furthermore, the positions in the cycle at which 5% CO_2 and dark treatments induce phase shifts which show no apparent relationship to the duration of the treatment are also similar. There are, nevertheless, two differences between the effects of 5% CO_2 treatments and dark treatments. The first concerns the magnitude of the phase shift induced by these treatments when they ended between approximately 2200 h and 0600 h on the third day of the experiment. A dark treatment ending between these positions in the cycle induced a phase shift of

approximately 8 - 10 h while a 5% CO_2 treatment resulted in a smaller phase shift of 4 - 6 h. The second difference concerns the lengths of the periods following exposure of the leaves to darkness and 5% CO_2. It was previously reported that a 4-h dark treatment lengthened the subsequent period in continuous light. No such effect occurred in response to a brief exposure to 5% CO_2. The period in leaves exposed to 5% CO_2 for 4 h (18.8 ± 0.21 h) was found not to be significantly different from the period in control leaves (18.7 ± 0.21 h). As a consequence, phase shifts induced by short duration 5% CO_2 treatments are stable, approximately the same amount of phase shift occurring in successive peaks.

In general, the circadian oscillator appears to be sensitive to 5% CO_2 treatments in those positions in the cycle at which it is also sensitive to low temperature and darkness. Maximum phase shifts were induced by these treatments when they ended a few hours after a peak in the rhythm of CO_2 exchange while little or no phase shift was induced by treatments ending in a trough. It was therefore of importance to establish whether or not 5% CO_2 treatments ending at various positions in the cycle shifted the phase in a similar direction to that achieved by exposure to low temperature and darkness. The principle of the procedure followed for determining the direction of the phase shift induced by 5% CO_2 treatments was that described previously for low and high temperature and darkness.

The continuous lines in Figs. 3.68 and 3.69 show the rhythms in leaves exposed to 5% CO_2; the darker of the two lines indicates the rhythm in leaves subjected to a 4-h treatment and the lighter line, that in leaves subjected to a 1-h treatment. In all experiments the 1- and 4-h treatments ended at the same time. The control rhythms in leaves maintained continuously in a stream of normal air are shown by the broken lines.

FIGURE 3.68. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* kept in light and a stream of normal air at 15°C by exposure to 5% CO_2 for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at midday as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (0800 h) and the second arrow the time at which the 1-h treatment began (1100 h). The broken line represents the rhythm in untreated control leaves. Ordinate: the rate of uptake (negative values) and output (positive values) of carbon dioxide by the leaves in $\mu g\ CO_2\ h^{-1}$ g (fresh weight)$^{-1}$. Abscissa: time of day, 0 = midnight.

FIGURE 3.68

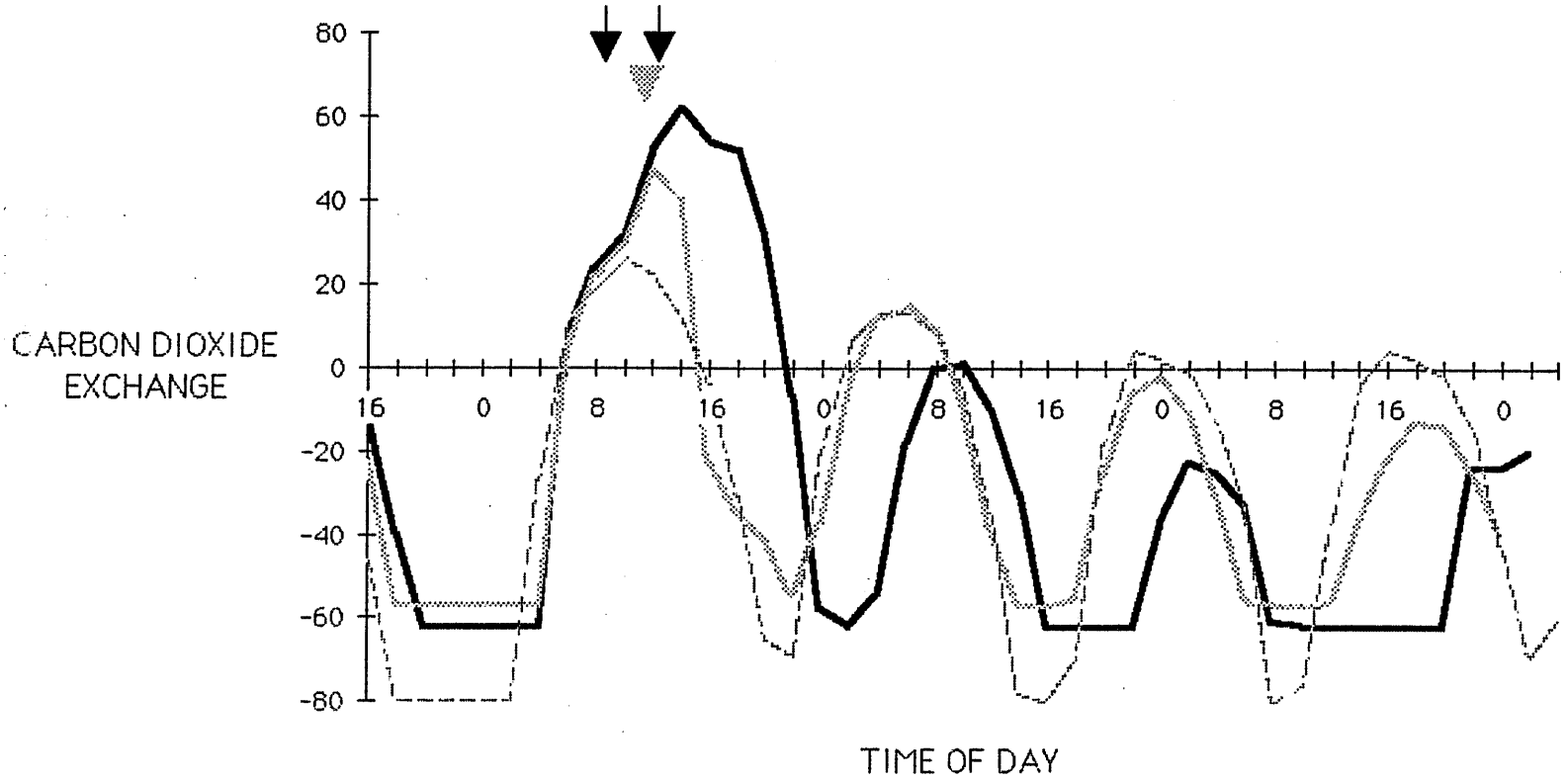

CARBON DIOXIDE EXCHANGE
80
60
40
20
0
-20
-40
-60
-80
16
0
8
16
0
8
16
0
8
16
0
TIME OF DAY

FIGURE 3.69. Phase shifts induced in the rhythm of CO_2 exchange in leaves of *Bryophyllum fedtschenkoi* in light and a stream of normal air at 15°C by exposure to 5% CO_2 for 4 h (dark continuous line) or 1 h (shaded continuous line). Treatments were timed to end at 1400 h as indicated by the third arrow. The first arrow indicates the time at which the 4-h treatment began (1000 h) and the second arrow the time at which the 1-h treatment began (1300 h). The broken line represents the rhythm in untreated control leaves. Presentation as for figure 3.68.

FIGURE 3.69

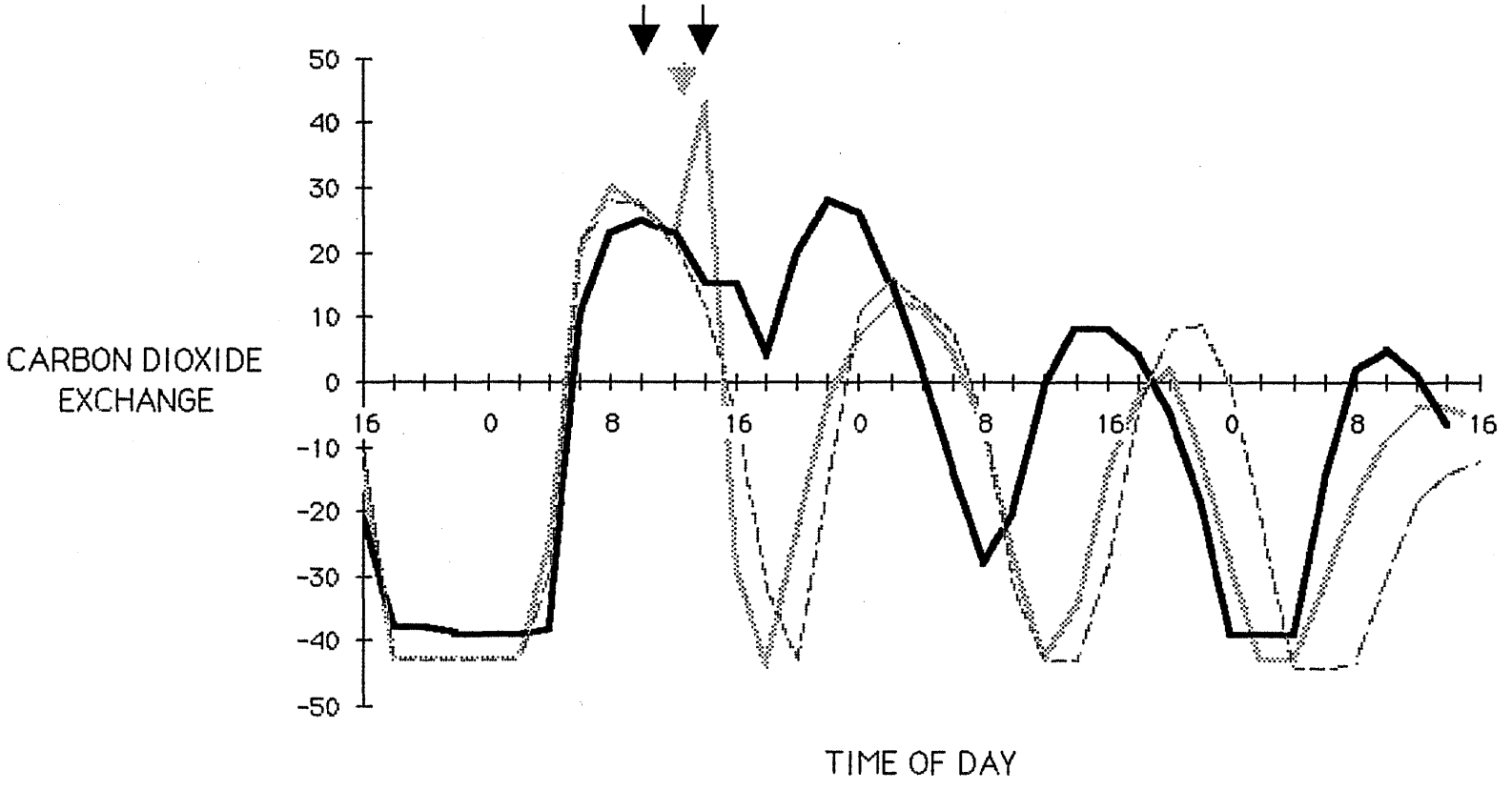
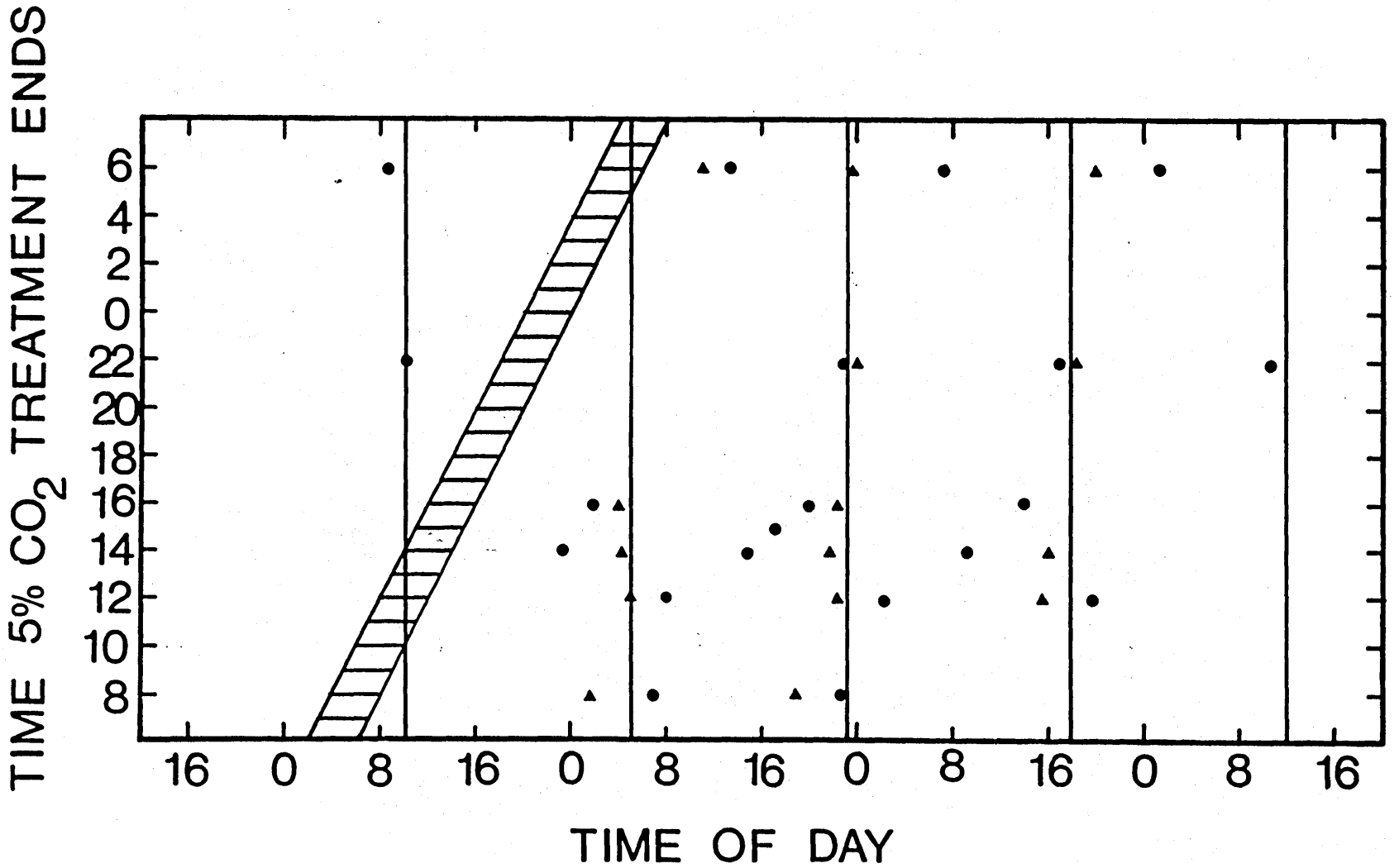

CARBON DIOXIDE EXCHANGE
50
40
30
20
10
0
-10
-20
-30
-40
-50
16
0
8
16
0
8
16
0
8
16
TIME OF DAY

FIGURE 3.70. Collated data for a series of experiments in which the cycle of CO_2 exchange in *Bryophyllum* leaves was scanned with a 4-h (●) or 1-h (▲) treatment during which time the leaves were exposed to 5% CO_2. Leaves were otherwise maintained in normal air and continuous light at 15°C. Times of occurrence of the peaks of untreated control rhythms are represented by the vertical lines. Points in any horizontal line show the mean times of occurrence of the peaks of the rhythms in at least two samples of leaves following a treatment at the time indicated on the ordinate. Treatment times are indicated by the shaded bar.


TIME 5% CO_2 TREATMENT ENDS
6
4
2
0
22
20
18
16
14
12
10
8
16
0
8
16
0
8
16
0
8
16
0
8
16
TIME OF DAY

In Fig. 3.68, the 1- and 4-h 5% CO_2 treatments ended at midday. The phase is unaffected by a 1-h exposure to 5% CO_2 at this position in the cycle but the 4-h treatment appears to delay the phase of the rhythm by approximately 3 - 4 h. A 1-h 5% CO_2 treatment ending at 1400 h appears to have only a very slight advancing effect on the phase of the rhythm, whilst a 4-h treatment appears to advance the phase by approximately 5 h (Fig. 3.69).

The results of a number of such experiments in which the leaves were subjected to 5% CO_2 for either 1 or 4 h at various positions in the cycle are shown in Fig. 3.70. Despite the somewhat limited data, it is clear that a 1-h, 5% CO_2 treatment has only a very slight effect on the phase of the rhythm. Nevertheless, it seems that in common with exposure to low temperature and darkness, brief exposures to 5% CO_2 between 1400 h and 1600 h advance the phase of the rhythm. A 5% CO_2 treatment ending at midday would appear to result in a phase delay. The phase would also have been expected to be delayed in response to a treatment ending at 0800 h on the second day of the experiment. The results in Fig. 3.70, however, suggest that a 1-h treatment advances the phase while the 4-h treatment results in a phase delay; considering the results presented in Fig. 3.67, it is likely that the phase shift represents a delay. If this view is correct then it follows that 5% CO_2, low temperature and dark treatments ending at various positions in the cycle shift the phase of the rhythm in a similar direction. It is nevertheless clear that before any definite conclusions could be made about the direction of the phase shift brought about by exposing leaves to 5% CO_2, a more extensive study would be necessary in which leaves were exposed to 5% CO_2 for 2 or 3 h and the phase shift induced compared with that induced by a 4-h treament.

The results presented in Figs. 3.68 - 3.70 also confirm an earlier finding that brief exposure of the leaves to 5% CO_2 has no effect on the subsequent frequency of

oscillation in normal air. In these experiments the period of the rhythm in leaves treated with 5% CO_2 for either 4 or 1 h was 17.7 ± 0.38 h and 17.6 ± 0.30 h respectively which was not significantly different from the control period of 17.9 ± 0.21 h. As previously mentioned, in experiments designed to establish the direction of the phase shift leaves were subjected to a slightly higher light intensity due to the necessary replacement of the fluorescent tube used to illuminate the leaves. This may explain the slightly shorter period recorded in leaves during these experiments. Relatively few experiments were carried out under the higher light intensity however, and the apparent shortening of the period may simply reflect the smaller amount of data available to determine period length. What is important here is the fact that 5% CO_2 does not appear to have an effect on the length of the period.

If 5% CO_2, darkness and low temperature exert their effects on the phase of the rhythm in a similar manner, then short exposures to 5% CO_2, in common with short exposures to darkness or low temperature should not be accompanied by a change in the gross malate status of the leaves. This prediction was tested by subjecting leaves to 5% CO_2 from 1000 h to 1400 h after they had been in a stream of normal air and continuous illumination for 18 h. Exposing leaves to 5% CO_2 at this position in the cycle was previously found to result in a large phase shift (see Fig. 3.67). Immediately after this treatment the cell sap of the leaves was extracted and the malate concentration compared with that in the sap from leaves maintained in a stream of normal air before being extracted at 1400 h. The results of two independent experiments and the average results of both experiments are shown in Table 10. In each experiment the malate content of 6 control, and 6 treated leaves was assayed in individual leaves, and the figures given represent the mean results .

TABLE 10

COMPARISON OF THE MALATE CONCENTRATION IN THE SAP FROM LEAVES MAINTAINED CONTINUOUSLY IN NORMAL AIR AND THOSE SUBJECTED TO 5% CO_2 FOR 4 h

EXP NO.	mM MALATE IN CELL SAP			
	CONTROL	5% CO_2-TREATED	t	S
1.	29.6 ± 2.58	32.1 ± 2.06	0.773	N.S.
2.	30.5 ± 2.53	31.5 ± 2.37	0.302	N.S.
1+2.	30.0 ± 1.71	31.8 ± 1.50	0.301	N.S.

These results show that there is no significant change in the malate status of the leaves following a 5% CO_2 treatment applied for 4 h from 1000 h to 1400 h. The lack of a difference in the malate content in the two samples of leaves could not be explained in terms of the leaves not being at the particular phase point normally expected at the times at which the treatment was applied. The rhythm in each sample of leaves was monitored prior to the extraction and in all cases the leaves were at the phase point where a 5% CO_2 treatment would normally result in a large phase shift. As predicted, the phase shift induced by a short 5% CO_2 treatment is not accompanied by a change in the gross malate status of the leaves.

The results in this section have therefore established the following points:

1. Prolonged exposure of the leaves to 5% CO_2 appears to inhibit the rhythm in CO_2 exchange, apparently by holding the oscillator at a fixed phase point in the cycle characterised by a leaf which is relatively rich in malate.

2. Exposing leaves to short periods of 5% CO_2 shifts the phase of the rhythm. The magnitude of the phase shift depends upon the position in the cycle at which the treatment is applied. The oscillator appears to be sensitive to 5% CO_2 at positions in the cycle at which it is also sensitive to low temperature and darkness.

3. The characteristics of the phase shifts induced by 5% CO_2 treatments are

remarkably similar to those induced by dark treatments.

4. In common with low temperature and darkness, the induction of a phase shift by a short 5% CO_2 treatment is not accompanied by an alteration in the gross malate status of the leaves.

4. DISCUSSION

The observation of a persistent rhythm of CO_2 exchange in the detached leaves of *Bryophyllum fedtschenkoi* maintained in continuous illumination, a stream of normal air and at a uniform temperature, confirms an earlier report by Wilkins (1984). Buchanan-Bollig (1984) has also reported the occurrence of such a rhythm in leaves of *Kalanchoë daigremontiana* when kept under similar conditions.

At 15°C the rhythm of CO_2 exchange in *B. fedtschenkoi* is very marked and persists for at least 10 days with an average period of 18.2 ± 0.16 h, one of the shortest periods ever to be recorded at this temperature (Sweeney and Hastings, 1960). The large amplitude, the very short period and the remarkable degree of persistence of the rhythm distinguishes it from the rhythm of CO_2 ouput occurring in leaves of this species maintained in darkness and an air-stream initially free of CO_2. The latter rhythm, which has been extensively studied by Wilkins (1959, 1960a and b 1962 a and b, 1967, 1973, 1983), is less marked (smaller amplitude) and persists for a maximum of 5 days with an average period of 23.8 ± 0.3 h at 16°C. This rhythm is thought to result from the periodic activity of PEPCase brought about by periodic accumulation of malate in the cytoplasm and its subsequent removal to the vacuole (Warren, 1964; Bollig and Wilkins, 1979). The rhythm in light and normal air is thought to arise from a similar basic mechanism. The following hypotheses have been suggested to explain the occurrence of these rhythms (Bollig and Wilkins, 1979; Wilkins, 1984). At the end of the 8-h photoperiod when the experiments begin the leaves contain relatively little malate and the rhythms begin by PEPCase becoming active. This activity leads to the accumulation of malate in the cytoplasm which eventually builds up to a level that is inhibitory to PEPCase. This stage is

represented by a peak in the rhythm of CO_2 output occurring in the dark, or in CO_2 exchange in the rhythm occurring in the light. Before a further period of CO_2 fixation can occur malate must be removed from the vicinity of PEPCase. In darkness and CO_2-free air, malate is thought to be removed from the cytoplasm by being pumped into the vacuole. When this occurs the activity of PEPCase is restored and a new cycle of CO_2 fixation can begin. In light and normal air malate removal is thought to occur by metabolic breakdown to produce pyruvate and CO_2, these breakdown products are removed by photosynthesis allowing the breakdown of malate to proceed to completion and the reappearance of PEPCase activity.

These hypotheses account for the fact that the rhythm persists for longer in light and normal air than in darkness and CO_2-free air. In the former the leaf cells are likely to begin each cycle of activity in virtually the same state, no limitation on the persistence of the rhythm is imposed by the gradual accumulation of malate in the leaf cells with time, as would occur in darkness (Wilkins, 1984). The length of time for which the latter rhythm can persist, on the other hand, is dependent upon the malate storage capacity of the vacuole. After about 5 days in continuous darkness the concentration of malate in the vacuole may attain a value which prevents any further net entry of malate. The malate would therefore remain in the cytoplasm where it would inhibit PEPCase which in turn would result in the abolition of the rhythm.

The shortness of the period of the rhythm in light and normal air compared to that in darkness and CO_2-free air has been explained by proposing that metabolic breakdown of malate in the light proceeds at higher rates than the trans-tonoplast pumping mechanism thought to operate in the dark (Wilkins, 1984).

Hitherto, the rhythm of CO_2 exchange exhibited by leaves of *Bryophyllum fedtschenkoi* in light and normal air has never been subjected to detailed

investigation. The present study has established the basic characteristics of this rhythm and, in addition, has provided information about the possible way in which the phase and period may be controlled by various environmental parameters. This information has in turn been of great value in assessing the validity of the hypothesis on the generation of the rhythm outlined above.

Whilst a number of interesting features of this rhythm have been revealed by this study, discussion of the results will focus on the main findings and the more peripheral ones will be discussed in less depth.

The circadian rhythm of CO_2 exchange exhibited by leaves of *Bryophyllum fedtschenkoi* maintained in continuous light and a stream of normal air operates within the approximate temperature range of between 10 and 30°C and is inhibited outwith this range. This finding is in accordance with reports on the effects of temperature on circadian rhythms in other organisms (Sweeney and Hastings, 1960).

A characteristic feature of most circadian rhythms is that the free-running period shows only a small dependence on ambient temperature, implying that the basic oscillator must incorporate some form of temperature-compensating mechanism. The rhythm reported in this thesis clearly possesses such a characteristic. The period of oscillation increased linearly with temperature from a minimum value of 15.7 ± 0.35h at 10°C to a maximum value of 22.3 ± 0.33 h at 30°C, indicating that the oscillator slows down with increasing temperature. The influence of temperature on the period is however, small, the average Q_{10} being 0.86, a value indicative of a considerable degree of temperature compensation. Nevertheless, the finding that the period is not independent of temperature is an important one since it implies that the underlying oscillator is of a metabolic nature and thus leaves little doubt that the rhythm is endogenous in origin.

Harris and Wilkins (1976) studied the influence of temperature on the rhythm of CO_2 output in leaves of this species in a CO_2-free air stream both in darkness and

under weak monochromatic radiation centred on 660 nm. The rhythms in the dark and the light reacted to temperature in the same way. Increasing the temperature from 15 to 25°C slightly increased the length of the period. In contrast to the results reported here, however, where the increase in period length was found to be linear over the entire temperature range from 10 to 30°C, these investigators found that the period at 30°C was significantly less than that at 25°C. This finding indicated that there was a maximum period at 25°C which decreased with either increasing or decreasing temperature. The results presented here, and those of Harris and Wilkins (1976) discussed above, contradict an earlier study on the effects of temperature on the rhythm in CO_2-free air and darkness where a small but significant reduction in the period was observed in response to increasing temperature within the range of 16 to 32°C (Wilkins, 1962b). The reason for such a discrepancy is uncertain but it was suggested (Harris and Wilkins, 1976) that the different conditions under which the plants were grown in the two studies may have resulted in differences in the water balance of the respective leaves which in turn altered the way in which the period responded to temperature. Water balance has been found to influence the period of other rhythms. Withholding water from *Phaseolus* seedlings, for example, lengthens the period of the leaf movement rhythm (Bünning and Moser, 1968). Such an explanation cannot however account for the different results reported here since the plants were grown under identical conditions to those used by Harris and Wilkins (1976). The different response of the period to temperature reported in the present investigation is perhaps more likely to reflect the different conditions under which the rhythm was monitored, that is, white fluorescent light and normal air as opposed to CO_2-free air and either darkness or weak monochromatic light. The temperature response of the period of the motility rhythm in *Euglena gracilis* differs under different conditions. (Brinkmann, 1966). In mixotrophic cultures the free-running period increases with increasing temperature in contrast to autotrophic cultures where the period is

independent of temperature within the range of 15 - 35°C.

Whilst it is true that most circadian rhythms run faster as the temperature is increased, the rhythm in *Bryophyllum* is not the only one in which the period is longer at higher than at lower temperatures. The first report of this phenomenon was in the rhythm of sporulation in *Oedogonium*(Bühnemann, 1955b) and was later found to occur in the rhythm of luminescence *Gonyaulax polyedra* (Hastings and Sweeney, 1959). The effect of temperature on the latter was similar to that reported by Harris and Wilkins (1976) in that the period increased with temperature up to a maximum at 27.6°C and then decreased between 27.6°C and 32°C.

It is interesting to note that the studies in *Oedogonium* , *Gonyaulax* , and *Bryophyllum* were all carried out in continuous light. These findings do not, however, permit generalisations to be made about the direction of the change in period with temperature under different lighting regimes since Grossenbacher (1939) reported that the period of the exudation rhythm in *Helianthus* seedlings decreased with increasing temperature in continuous light and the rhythms of CO_2 output in *Bryophyllum* (Harris and Wilkins, 1976) and of cell division in *Gonyaulax* (Hastings and Sweeney, 1959) increased with increasing temperature in the dark. The validity of the results of the latter study is, however, questionable since only one period was measured at each temperature and only one experiment appears to have been carried out.

In a few organisms the period has been found to be completely independent of temperature. In *Paramecium* , for example, no detectable change in the period of the mating rhythm was observed between 17 and 30°C (Ehret, 1959). In a number of other organisms the period appears to be remarkably temperature dependent. Failure to recognise that temporary instability in the period often accompanies a change in temperature was the basis for the conclusion of many early studies that circadian periods were dependent on temperature (Bünning, 1973; Sweeney and Hastings, 1960). However, even when those so-called transient cycles are eliminated there are a number of rhythms where temperature has been found to

have a relatively large effect on the period. Bünning (1974) has suggested that temperature compensation may have come about by Darwinian selection. Selection pressure would be particularly strong in temperate regions with large seasonal variations in temperature but only weak or absent in the tropics where little seasonal fluctuation in the temperature occurs. In support of this argument he cites the rhythm of odour production in the tropical plant *Cestrum nocturnum* where in continuous light the period is 24 h at 20°C, 27 h at 17°C, and 31 h at 14°C (Overland, 1960) and that of leaf movement in *Phaseolus mungo* where a period of 32 ± 0.8 h was recorded at 17°C and 24.6 ± 0.2 h at 32°C (Mayer, 1966).

In most organisms the difference between the maximum and minimum period at different temperatures is of the order of 1 - 2 h, but Bünning (1974) notes than in *Gonyaulax polyedra*, a tropical alga, the difference between the maximum period at 32°C and the minimum at 15°C is about 4 h. *Bryophyllum fedtschenkoi*, is a desert plant and a difference of almost 7 h exists between the maximum period at 30°C and the minimum period at 10°C when the rhythm is recorded in normal air and light. It is clear, however, from the foregoing discussion, that the influence of temperature on the period of a rhythm depends upon the conditions under which it is measured. In CO_2-free air and darkness a difference of only 1 h exists between the maximum and minimum period of the rhythm in *Bryophyllum*. (Harris and Wilkins, 1976). These investigators also found that the rhythm of CO_2 output recorded in weak monochromatic light showed a greater dependence on temperature than the dark rhythm. The Q_{10} values of the rhythms being 1.06 and 1.09 respectively. It is interesting to note that all the rhythms in which temperature has a relatively large influence on the period have been recorded in continuous light. It would therefore appear that valid comparisons of the influence of temperature on the period of circadian rhythms can only be made between rhythms which have been recorded under the same conditions. Failure to realise that the period of a particular rhythm may respond differently to temperature

under different environmental conditions, and the fact that the rhythm of leaf movement in several tropical species is virtually temperature independent (Mayer, 1966) makes Bünning's hypothesis difficult to sustain.

The time which elapses between the beginning of an experiment and the time taken to reach a defined phase point, usually the first peak of a rhythm, has also been found to be influenced by temperature in some organisms. This time interval, which has been termed the transient (Wilkins, 1960 a and b), has been found to show a greater dependence on temperature than the free-running period. A few of these studies were described in the introduction to this thesis. In the present study no clear-cut relationship between temperature and the length of the transient was found. The time which elapsed between the onset of the experiment and the occurrence of the first peak of the rhythm at 20 and 25°C was significantly greater than the time taken at 12.5, 15, and 18°C. The transient at 10°C, however, was not found to be significantly different from that at any other temperature. These results contrast with two earlier reports on the effects of temperature on the transient of the rhythm of CO_2 output in *B. fedtschenkoi* leaves in CO_2-free air and darkness where a decrease was observed on increasing the temperature from 16 to 32°C (Wilkins, 1962a). In addition to confirming these results, the later study of Harris and Wilkins (1976) revealed that the transient of the rhythm which occurred in weak monochromatic radiation also decreased with increasing temperature. In contrast to the period however, which showed a greater dependence on temperature in the light than in the dark, the transient appeared to be less dependent on temperature in the light than in the dark as indicated by the temperature coefficients (transient at 16°C/transient at 26°C) of 1.2 and 1.3 respectively.

The results presented here may be complicated by the fact that the leaves usually experienced a temperature change on being transferred from the growth cabinet to the experimental temperature. In order to make a more accurate assessment of the effect of temperature on the transient more detailed experiments

would need to be conducted in which plants in the growth cabinet were held at the experimental temperature for a few days before the transfer of the leaves to the leaf chambers occurred.

An interesting and somewhat surprising result to emerge from this investigation was that at certain constant ambient temperatures the free-running period was rather unstable. In fact, complete period stability was observed only in leaves held at 15, 20 and 28°C. At 18°C the steady-state period was reached only after the first cycle had been completed. As previously mentioned, a temporary instability in the period often follows a temperature change. Pittendrigh *et al.* (1958) have termed these unstable cycles transients. This definition of the term obviously differs from that used by Wilkins (1960a). The instabilities of the period reported here however, cannot be explained simply in terms of transient cycles occurring in response to the temperature change which the leaves experienced on being transferred from the growth cabinet to the experimental temperature. Marked instability of the period was observed in leaves held continuously at 25°C despite the fact that the temperature of the cabinet from which the leaves were transferred was 25°C. Moreover, it would appear that at 10 and 25°C the period never becomes stable.

There appears to be no simple explanation for these results. It is possible that while the rhythm can operate within the approximate temperature range of 10 - 30°C, the temperature compensating mechanism operates efficiently only within the much narrower range of 15 - 20°C. Such an explanation does not account for the fact that the period is stable at 28°C or that the first period at 18°C appears to be significantly shorter than all successive periods. These latter results may reflect the limited amount of data available for analysing the period at these temperatures; this point would merit further investigation.

The mechanism by which temperature affects the period of circadian rhythms has never been established in any organism. Temperature compensation has been suggested to be the result of the speeding up of some reactions and the slowing

down of others with the result that little overall change in the period occurs in response to changes in temperature (Bünning, 1973). In *Bryophyllum tubiflora*, Brandon (1967) has reported that the activity of the malate synthesising enzymes and those involved in malate breakdown have different temperature optima. Such a finding was not however confirmed in *Kalanchöe daigremontiana* (Nott and Osmond, 1978, unpublished data, see Osmond and Holtum, 1981), a point which will be discussed more fully later.

The recently discovered *frq9* mutant of *Neurospora crassa* (Loros *et al.*, 1986) may provide a useful tool for the study of the mechanism by which temperature influences the length of the period since this mutant shows complete loss of temperature compensation. Elucidation of such a mechanism cannot realistically be achieved without a much better understanding of the reactions involved in the generation of the underlying circadian oscillator.

The amplitude of circadian rhythms is markedly affected by temperature. However, very few studies have concentrated on the influence of temperature on this parameter of the rhythm since it is highly unlikely to help in elucidating the time-keeping mechanism. In *Euglena gracilis* the amplitude of the phototaxis rhythm is reduced by low temperature (Bruce and Pittendrigh, 1956) whilst that of the luminescence rhythm in *Gonyaulax* decreases with increasing temperature (Hastings and Sweeney, 1957, 1959). In the present investigation maximum amplitude of the CO_2 exchange rhythm occurred between 15 and 20°C and decreased with either increasing or decreasing temperature. Despite the fact that the amplitude of the rhythms in *Gonyaulax* and *Bryophyllum* respond differently to temperature, a striking similarity between the rhythms in these two organisms is observed when they are maintained at 32 and 30°C respectively. In both organisms a period of approximately 3 days elapses between the beginning of the experiment and the occurrence of a well defined peak. Moreover, at these temperatures both rhythms have a low amplitude and die out after only 3 cycles. Quite what this finding means in terms of the biochemical events underlying the rhythms in these

organisms cannot be assessed at this stage.

The precise temperature range within which the rhythm of CO_2 exchange occurs was determined in this study to within a limit of 5°C. The upper and lower limits which permit oscillation lie between 30 and 35°C and 5 and 10°C respectively because no rhythm was ever detected in leaves held at either 35 or 5°C.

The nature of the inhibition of the rhythm at high and low temperatures is, however, fundamentally different. At high temperatures (i.e. 35 and 40°C) the rhythm was inhibited in such a way that little or no fixation of CO_2 occurred. A quite different pattern of CO_2 exchange was observed in leaves held at low temperatures. After the initial increase in the rate of CO_2 uptake, leaves held at 5 or 2°C decreased their rate of uptake to a value of approximately 30 - 40 $\mu g\ CO_2\ h^{-1}$ g (fresh weight)$^{-1}$ and continued to fix CO_2 at about this rate thereafter. This lower constant rate of fixation appeared to be entirely attributable to photosynthesis since CO_2 uptake was immediately replaced by CO_2 output on transferring the leaves to darkness. The finding that CO_2 is fixed at all at low temperatures is clearly an important one since it implies that both the malate synthesising enzymes and those involved in the C3 pathway are capable of operating at temperatures as low as 2°C.

The possibility that prolonged exposures to extreme temperatures had either damaged the leaves or, that these treatments had only inhibited the overt rhythm while the basic oscillator continued to operate normally was eliminated by experiments in which leaves held at 40 or 2°C for a few days were transferred to 15°C.

Transferring leaves from 40 to 15°C initiated a rhythm which always began with a burst of CO_2 uptake and persisted for many days thereafter. Regardless of the time at which the temperature change was made, the first peak of the newly initiated rhythm occurred approximately 15 h later. This finding implies that high

temperature inhibits the rhythm by holding the basic oscillator at a fixed phase point in the cycle from which it is released on transferring the leaves to 15°C. A similar conclusion was reached by Wilkins (1962b). Exposing leaves of *Bryophyllum* to 36°C inhibited the rhythm of CO_2 output occurring in leaves maintained in darkness and a CO_2-free air stream. Lowering the temperature to 16°C initiated a rhythm and set the phase in a similar manner as reported here.

Low temperature also appeared to inhibit the rhythm by holding the basic oscillator at a fixed phase point in the cycle. The fixed phase point to which low temperature held the oscillator, however, appeared to be at the opposite extreme in the cycle to that at which it was held by high temperature. Transferring leaves from 2 to 15°C also initiated a rhythm, but, in contrast to the rhythm which began after transfer from 40°C with an increase in the rate of CO_2 uptake, this rhythm began by the leaves immediately decreasing their rate of CO_2 uptake. The first peak of the rhythm always occurred approximately 4 h after the end of the low temperature treatment irrespective of the time of day at whch the transfer from 2 to 15°C occurred. The phase of the newly initiated rhythm is therefore wholly determined by the time at which the low temperature treatment ends.

There have been several other reports of the effects of prolonged periods of extreme temperatures on circadian rhythms. For example, in *Gonyaulax polyedra*, the rhythm of luminescence is inhibited at 11°C but begins immediately on restoring the temperature to 20°C, the phase being determined by the time in the cycle at which the low temperature treatment ends (Njus, McMurry and Hastings, 1976). A similar finding has been made for the rhythm of petal movement in *Kalanchoë blossfeldiana* (Oltmans, 1960). Kondo and Tsudzuka (1980a and b) have studied the effects of prolonged periods of 4.5 and 39°C on the rhythm of K^+ uptake exhibited by *Lemna gibba* in continuous light. In contrast to the results presented here, these investigators found that the rhythms which commenced on transferring organisms from either 4.5 or 39°C to a temperature which permitted

oscillation, always began with a peak in the K^+ uptake rhythm. This finding implied that high and low temperatures inhibited the rhythm by forcing the basic oscillator to, and holding it at, the same fixed point in the cycle.

The results reported in this thesis are therefore by no means the first demonstration that prolonged exposure to extreme temperatures inhibits oscillation by forcing the basic oscillator to, and holding it at, a fixed phase point in the cycle. A wholly new finding to emerge from this investigation however, is that in *Bryophyllum fedtschenkoi* , high and low temperatures prevent oscillation by forcing the basic oscillator to phase points in the cycle which differ by 180^0. Furthermore, it has been possible to characterise these fixed phase points in terms which are fully compatible with the hypothesis advanced to account for the generation of this rhythm (Wilkins, 1984).

Since scarcely any CO_2 was fixed by leaves held at 40^0C, and fixation began immediately the leaves were transferred to 15^0C, it was predicted that the fixed phase point at which high temperature held the oscillator must represent a state in which the leaves contained little or no malate. On the other hand, the initial substantial period of CO_2 fixation observed in leaves held at 2^0C would be expected to lead to the accumulation of malate in the leaf cells, and the rapid decrease in the rate of CO_2 uptake and even CO_2 emission which occurs on transferring leaves to 15^0C presumably reflects the decarboxylation of malate which would have to occur to enable PEPCase to operate. It was predicted, therefore, that the phase point at which low temperatures held the oscillator represented a state in which the leaf cells contained relatively large amounts of malate.

These predictions were entirely borne out by experimental determination of the malate status of the leaves. The concentration of malate in the cell sap of leaves exposed to 40^0C for various lengths of time remained low at values which varied between approximately 7 and 20 mM. There was no evidence to suggest that either net malate synthesis or breakdown occurred during the high temperature

treatment.

At 2°C large amounts of malate accumulated in the cell sap, the pattern of accumulation being exactly as predicted from the CO_2 exchange curve. The malate concentration in the cell sap increased from an initial value of about 15 mM to reach half its maximum concentration 8 h later, a time which corresponded with the time at which maximum rates of uptake of CO_2 occurred. Thereafter the rate of malate accumulation declined in parallel with the decrease in the rate of CO_2 uptake. Approximately 24 h after the leaves had been placed at 2°C the malate concentration in the cell sap reached its maximum value of about 50 - 60 mM, corresponding with the time at which the rate of CO_2 fixation became constant. No change in the malate content of the leaves was observed after this time. The change in the malate concentration of the cell sap was paralleled by a change in pH.

These findings, together with the CO_2 fixation pattern observed in leaves held at high and low temperatures, and the way in which the rhythm begins on transferring leaves to 15°C clearly indicate that the inhibition of the rhythm at high temperature is associated with the leaf having a malate-low status and at low temperature by the leaf having a malate-rich status.

Although net malate synthesis is inhibited by high temperature it was shown that malate breakdown could proceed even at 40°C. When leaves which had been maintained at 2°C for 44 h were transferred to 40°C they showed no tendency to retain the the high levels of malate which had accumulated during the low temperature treatment. After approximately 2 h, the concentration of malate in the cell sap of leaves transferred from 2 to 40°C gradually decreased from about 50 mM immediately prior to the transfer to about 25 mM 8 h later. No further reduction in malate content occurred when the 40°C treatment was extended for up to 24 h. The declining malate level was reflected by a gradual increase in the pH of

the sap. The reason for the 2-h delay between transferring leaves from 2 to 40°C and the onset of malate breakdown is uncertain. It is possible however, that the enzyme system involved in this reaction takes time to adapt to such an extreme change in temperature.

As expected, the rhythm which began when leaves which had been exposed sequentially to 2°C for 44 h and 40°C for 4 h were transferred to 15°C was not 180° out of phase with the rhythm which was initiated on transferring leaves directly from 2 to 15°C at the same time. The first peak in the 40°C-treated leaves occurred approximately 18 h after the transfer to 15°C, 3 h later than the usual time of occurrence of the peak in leaves which were held continuously at 40°C before being transferred to 15°C. Since the 4-h 40°C treatment only partially reduced the high levels of malate which had accumulated at 2°C, the later occurrence of the first peak in the former case could be explained by the extra time required at the end of the high temperature treatment for the leaves to reduce their malate concentration to a value which enabled PEPCase to operate. Since an 8-h 40°C treatment is apparently of sufficient duration to reduce the high levels of malate which accumulate at 2°C to a value which is consistent with that found in leaves at the end of the normal 8-h photoperiod, it was somewhat surprising to find that the rhythm which began on transferring leaves treated in this way to 15°C was not exactly 180° out of phase with the rhythm in leaves transferred directly from 2 to 15°C at the same time. The first peak in leaves which had been exposed sequentially to 2°C for 44 h and 40°C for 8 h, occurred approximately 17 h after the transfer to 15°C, 2 h later than the usual time of occurrence of the peak of the rhythm in leaves transferred directly from 40 to 15°C.

There are two possible explanations for this unexpected result. First, only two experiments were carried out to determine the phase of the rhythm in leaves transferred to 15°C after having previously been exposed to 2°C for 44 h and then 40°C for 8 h and, in each experiment only one sample of leaves was treated in this way. Many more data were available to determine the phase of the rhythm in

leaves transferred directly from 40 to 15°C. Second, the malate concentration in leaves extracted from plants in the growth cabinet at the end of the normal 8-h photoperiod varies between a minimum value of approximately 5 mM and a maximum value of approximately 25 mM. It is, therefore, possible that the malate concentration of leaves used in experiments involving a single temperature change from 40 to 15°C was at the lower end of this scale whilst that in leaves exposed first to 2°C and then to 40°C for 8 h before being transferred to 15°C may have been at the upper end of the range. It is conceivable that the rate of malate synthesis is determined by the amount of malate present when the temperature is lowered to 15°C, lower levels of malate leading to faster rates of synthesis hence reducing the time required to reach the first peak in the rhythm.

These possibilities could be resolved by more extensive experiments involving sequential exposure of the leaves to 2, 40 and 15°C, but the important point to be established by this study was that malate breakdown took place at 40°C.

The way in which high and low temperature could lead respectively to malate-poor and malate-rich states is uncertain. Three possibilities must be considered with respect to the way in which high temperature could result in a leaf having a malate-low status. Firstly, high temperature may act by closing the stomata securely thereby preventing gas exchange between the leaf and the atmosphere. Indeed, a marked reduction in nocturnal stomatal opening in *Kalanchoë blossfeldiana* occurs in response to high night temperatures (Nishida, 1963). More recently, however, Buchanan-Bollig (1984) has reported that the transpiration rate in *Kalanchoë daigremontiana* is uniformly high when leaves are maintained in continuous illumination at 28.8°C indicating that the stomata remain open during such a treatment. It is significant to note that in the present investigation a marked loss in the turgidity of the leaves was observed after they had been maintained at 40°C for a few days. This finding indicates that transpiration must have occurred and therefore the stomata would have been open for at least some of the time during the 40°C treatment. It seems unlikely therefore,

that high temperature acts via stomatal closure to prevent malate synthesis. A second possibility is that high temperature greatly increases the turnover of malate such that no net accumulation occurs. This is an unlikely explanation, however, in view of the large number of reports indicating that malate synthesis is inhibited by high temperatures and stimulated by low temperatures (see Kluge and Ting, 1978). The simplest and most plausible explanation for the effects of high and low temperature on the pattern of CO_2 exchange and malate accumulation reported here is that high temperature inhibits PEPCase, thereby preventing malate synthesis while low temperature inhibits malic enzyme thus preventing malate breakdown. If such an interpretation is correct then PEPCase and malic enzyme may be expected to have different temperature optima.

There appear to have been only two studies in which the effects of temperature on the enzymes responsible for malate synthesis and breakdown have been investigated simultaneously. The first of these investigations was that by Brandon (1967) who reported that the optimum temperature of PEPCase from *Bryophyllum tubiflora* was 35°C whilst the optimum temperature for malic enzyme was not even reached at 53°C. As previously mentioned these results were not confirmed by Nott and Osmond (unpublished data, see Osmond and Hotum, 1981), who found no significant difference in the temperature responses of PEPCase and malic enzyme from *Kalanchoë daigremontiana*. The difference in the results of these two studies may have arisen from the fact that crude extracts were used in the former whereas measurements made with *Kalanchoë* were based on the *in vitro* properties of the purified enzymes.

The effects of temperature on purified PEPCase from leaves of *Bryophyllum fedtschenkoi* have been investigated (Jones, Wilkins, Coggins, Fewson and Malcolm, 1978). These authors reported the occurrence of a reversible temperature-dependent inactivation of the enzyme between 30 and 45°C. The small amount of fixation carried out by leaves held at 35°C in the present investigation may indicate that *in vivo* this temperature is at or very near the maximum

temperature at which PEPCase can operate. It is equally possible, however, that this low level of fixation is due entirely to photosynthesis.

Whether or not the temperature properties of purified enzymes or even crude extracts *in vitro* reflect those of the enzymes *in vivo* is very uncertain. This point was clearly illustrated in a recent study of the effects of temperature on purified and crude extracts of PEPCase from *Kalanchoë daigremontiana* (Buchanan-Bollig, Kluge and Muller, 1984). These authors reported that the temperature optimum for this enzyme depended upon a number of factors including the pH of the assay mixture, the substrate concentration and various metabolic effectors. At saturating substrate concentrations a temperature optimum of 40 - 42°C was observed, but this value could be reduced to 20°C if the enzyme was assayed in the presence of malate at low substrate concentrations.

It would appear of more value therefore to compare the results reported in the present study with other investigations in which the activities of the enzymes involved in malate synthesis and breakdown have been studied indirectly by examining the effects of temperature on these processes. The first investigation of this nature was carried out using the detached leaves of *Sedum praealtum* (Bennet-Clark,1933). When leaves were maintained in darkness at 27°C the acid which first acccumulated gradually declined to attain a low stable value. When leaves were maintained at 3°C, however, malate accumulated to a maximum concentration and was retained at this level as long as the leaves were held at such a temperature. Transferring leaves from high to low temperatures resulted in an increase in the level of malate and, conversely, a decrease in the malate content of the leaves was observed when they were transferred from low to high temperature. These results are entirely consistent with those reported here in that they indicate that high temperature leads to a malate-poor leaf whilst low temperature leads to a malate-rich leaf.

There have been a few reports of the inhibition of net dark CO_2 fixation by low

temperatures. For example, net CO_2 uptake by leaves of *Kalanchoë daigremontiana* was found to be inhibited at 5°C (Nurenbergk, 1962). In view of the results presented in this thesis however, and those of Dinger and Patten (1974), who found that dark CO_2 fixation could operate normally in *Echinocereus* at 5°C, it appears that variation with respect to temperature sensitivity of dark CO_2 fixation exists between species. Nevertheless, the results presented in this thesis do suggest that the rate of CO_2 fixation may be slowed down by low temperature. Maximum malate concentration in the cell sap did not occur until approximately 24 h after the leaves had been subjected to 2°C while the malate levels in leaves placed in continuous light at 15°C at the end of the photoperiod reached a maximum value approximately 12 h later. Jones and Mansfield (1972) have also suggested that the delayed appearance of the the first peak of the CO_2 compensation rhythm in leaves of *Bryophyllum fedtschenkoi* at 10°C compared to that at 15°C may be attributable to a longer acidification period at the lower temperature.

Brandon (1967) studied the temperature properties of PEPCase and malic enzyme in *Bryophyllum tubiflorum* by measuring the amount of malate produced or broken down at different temperatures. His results clearly indicated that low temperature favoured malate synthesis whilst malate decarboxylation predominated at high temperatures.

A reduction in the rate of malate synthesis in leaves of *Kalanchoë daigremontiana* held at high temperatures has been reported by Medina and Osmond (1982). This study is particularly interesting because it showed that the temperature response of the enzymes involved in the synthesis and breakdown of malate depend upon the temperature regime under which the plants had previously been grown. Plants which had been maintained at a daytime temperature of 25°C and a night-time temperature of 15°C exhibited high rates of malate synthesis at 8 and 15°C but this rate was markedly reduced at 24°C. In contrast, plants which were

maintained at 34°C during the photoperiod and 24°C at night showed high rates of acid synthesis at 24°C but very much reduced rates at 8°C. Since the plants used in the present study were maintained in a 25:15°C temperature regime it is perhaps not surprising that net malate synthesis was inhibited at 40°C. The investigation of Medina and Osmond has, however, further consequences for future investigation of the rhythm of CO_2 exchange in *Bryophyllum fedtschenkoi* since it has opened up the exciting possibility of altering the patterns of malate accumulation and CO_2 exchange observed in leaves held at 40 and 2°C by growing plants under different temperature regimes. If this proposal does in fact turn out to be possible, and it could be further demonstrated that altering the patterns of CO_2 exchange and malate accumulation in turn alters the phase of the rhythm initiated on transferring these leaves to 15°C, then this would provide good evidence to suggest that in *Bryophyllum fedtschenkoi* malate accumulation and breakdown may be an intregal part of the basic circadian oscillator.

Further support for the hypothesis that malate synthesis and breakdown are intimately involved in the generation of the rhythm of CO_2 exchange was provided by the investigation of the effects of darkness on the rhythm.

Prolonged exposure of the leaves to darkness inhibits the rhythm and results in the accumulation of large amounts of malate in the cell sap. Furthermore, this treatment controls the phase of the rhythm which is initiated on transferring leaves into light in a predictable manner.

The pattern of CO_2 exchange exhibited by leaves maintained in continuous darkness and a stream of normal air at 15°C reported here is essentially similar to that reported by Wilkins (1984) and more recently by Nimmo *et al* (1987). The CO_2 which is fixed by the leaves during the first 16 h in continuous darkness results in the malate concentration of the cell sap increasing from approximately 15 - 20 mM at the start of the experiment to approximately 50 - 55 mM 16 h later. Thereafter the

rate of CO_2 output and the concentration of malate in the cell sap slowly declined but appeared to reach constant values after approximately 40 - 48 h in continuous darkness.

The inhibition of the rhythm must represent a true inhibition of the basic oscillator because transferring leaves from darkness into continuous light initiates a rhythm and sets the phase. The first peak of the newly initiated rhythm always occurs approximately 24 h after the end of the dark treatment, irrespective of the time of day at which the leaves were placed in continuous light. This finding clearly indicates that darkness inhibits the rhythm by holding the basic oscillator at a fixed phase point in the cycle.

Darkness is known to inhibit rhythms and set their phase in a number of other organisms. A few of these studies were outlined in the introduction, suffice it to mention here that none discussed the possible nature of the phase point at which the oscillator was held by darkness in terms of the biochemical or molecular events underlying the generation of the oscillation.

Wilkins (1984) explained the inhibition of the rhythm of CO_2 exchange in normal air and darkness by proposing that the amount of malate accumulated in the leaf cells during the first 16 h in continuous darkness was so high that it could not be completely removed from the cytoplasm by being pumped to the vacuole. Thus, it remains in the cytoplasm where it inhibits PEPCase and results in the abolition of the rhythm.

The results presented here do give partial support for this view in so far as darkened leaves do accumulate large amounts of malate. But this investigation, and indeed that of Nimmo *et al.* (1987), have also indicated that leaves of *Bryophyllum fedtschenkoi* are able to break down at least some of the malate which accumulates during the initial period in darkness. Loss of malate from leaves maintained in prolonged darkness has been reported by several other investigators (e.g. Kluge Bohlke and Queiroz, 1981; Buchanan-Bollig, 1984) and at least the initial loss is

known to occur by decarboxylation of malate (see Kluge and Ting, 1978). When leaves of *Kalanchoë daigremontiana* are maintained in continuous darkness, the internal CO_2 concentration rises sharply at a time which would correspond with the onset of the normal light period (Kluge *et al.*, 1981). The increase in CO_2 concentration is accompanied by a decrease in the malate concentration of the cell sap and shortly afterwards by the closure of the stomata.

In the present investigation the decline in malate content which began after the leaves had been in darkness for 16 h appeared to occur for only a limited period of time. After approximately 48 h in darkness, CO_2 was given out by the leaves at a constant rate; the malate concentration and pH of the cell sap also appeared to remain constant after this time suggesting that little or no further net decarboxylation of malate was occurring. Presumably after 48 h the CO_2 produced by decarboxylation of malate had reached a concentration where it prevented any further malate breakdown. High ambient CO_2 concentrations have been found to inhibit malate decarboxylation in four other species of CAM plants (Nishida, 1977; Fischer and Kluge, 1985); this point is discussed more fully below.

Nimmo *et al.* (1987) found, that the malate content in leaves of *Bryophyllum fedtschenkoi* continued to decline even after they had been in darkness for 120 h. If prolonged exposure of the leaves to darkness does indeed lead to high internal CO_2 concentrations, then it is unlikely that this further decline is due to malate decarboxylation since the internal CO_2 concentration would be too high to allow such a reaction to occur. After a long period in darkness the energy supply of the leaves must be extremely low and it is possible that malate may move into the mitochondria where it is used in the citric acid cycle as has been demonstrated in maize roots (Lipps and Beevers, 1966a and b).

The experiments which involved transferring leaves from dark to light revealed that the oscillator is held at a fixed phase point in the cycle during the

dark treatment. It is therefore necessary to consider what this fixed phase point would be in terms of the malate status of the cell sap. Leaves were transferred to continuous light after they had been in darkness for various lengths of time from 38 to 80 h. Making the assumption that malate accumulation and breakdown in these leaves followed a similar pattern to that in the leaves actually used to determine the malate status, then by 38 h decarboxylation of malate should have almost ceased. This is a reasonable assumption to make because in each experiment the rate of CO_2 output was constant before the leaves were transferred to continuous light indicating that net breakdown of malate had probably stopped. Thus, the fixed phase point to which darkness drove the oscillator in these experiments must have represented a stage in which the leaves contained a moderately high level of malate. After the end of the dark treatment the leaves presumably have first to reduce this concentration to a level which would enable PEPCase to operate. The rhythm initiated on transferring leaves from dark to light would therefore be expected to begin in a similar manner to that which is initiated on transferring leaves from 2 to 15^{0}C in the light, that is by a marked increase in the rate of CO_2 output or decrease in CO_2 uptake. The 6 - 8 h period which elapses between transferring leaves from darkness to light and the onset of net CO_2 uptake probably reflects the time necessary for the leaves to break down the malate which is present at the end of the dark period. No increase in the rate of CO_2 output accompanies the change from dark to light presumably because the CO_2 derived from malate decarboxylation is immediately consumed in photosynthesis. The first peak of the rhythm which is initiated after transferring leaves from darkness to light is not therefore detectable.

It is interesting to compare the phase of the rhythm which begins on transferring leaves from light to dark with the phase of that which is initiated in leaves transferred from 2 to 15^{0}C in the light. It would be predicted that the time

between transferring leaves from dark to light and the occurrence of the first trough in the rhythm would be shorter than the time taken for leaves to reach the first trough after a 2°C treatment in the light because at the end of the dark period leaves contain less malate than those at the end of a low temperature treatment. Maximum rates of CO_2 fixation would therefore be expected to be attained earlier in the former. On making such a comparison, however, it is found that the first trough of the rhythm initiated by transferring leaves from dark to light occurs about 3 h later than that in the rhythm which begins after a low temperature treatment (See Figs. 3.17, 3.18, 3.48 and 3.49). This somewhat surprising result is difficult to explain. One possibility is associated with the energy supply of the leaves after a prolonged period in darkness. It is now well established that the PEP for malate synthesis is derived from starch via the glycolytic pathway (Kluge and Ting, 1978; Ting, 1985) and it has been demonstrated several times that malate and starch accumulation show a reciprocal relationship (see Kluge and Ting, 1978). In continuous darkness the starch content of *Kalanchoë daigremontiana* leaves remains very low (Buchanan-Bollig, 1984). It may therefore be envisaged that the time required for leaves previously maintained in continuous darkness to reach a trough in the rhythm after transfer to light will be limited by the time necessary for photosynthesis to build up enough starch which can in turn be broken down to PEP required for malate synthesis.

In summary, it is proposed that darkness inhibits the rhythm of CO_2 exchange in a similar manner to low temperature by inhibiting malate breakdown. Since there is no evidence from the results in the present investigation to suggest that leaves can break down malate at 2°C, it is necessary to propose that the inhibition of the rhythm by low temperature is simply due to the fact that malic enzyme is incapable of operating at 2°C. Darkness on the other hand inhibits malic enzyme indirectly by allowing the malate which initially accumulates to be broken down until the enzyme is eventually inhibited by high internal CO_2 concentrations

which persist in the absence of photosynthesis.

If this hypothesis is correct an important prediction can be made. High ambient CO_2 concentrations would be expected to inhibit the rhythm in illuminated leaves in a manner similar to that achieved by low temperature and darkness, that is by inhibiting malic enzyme thereby resulting in a leaf which contains relatively high levels of malate. The malate concentration in the sap pressed from such leaves, the inhibition of the rhythm, as well as its reappearance and phase when the CO_2 concentration is reduced, have all been examined, and the results provide substantial support for the above hypothesis.

Exposing leaves to 5% CO_2 appears to inhibit rhythmic CO_2 fixation by forcing the basic oscillator to, and holding it at, a fixed phase point in the cycle represented by a leaf which contains relatively large amounts of malate. The evidence for this conclusion is provided by the pattern of malate accumulation in the cell sap and the phase of the rhythm initiated on transferring leaves from 5% CO_2 to normal air. In continuous light at 15^{0}C and in an air stream containing 5% CO_2, the malate concentration of the cell sap increased from approximately 20 mM at the beginning of the experiment to reach a value of 40 - 50 mM 16 h later. No further change in the malate concentration of the sap was observed after this time. Transferring leaves from 5% CO_2 to normal air initiated a rhythm, the first peak of which always occurred about 23 h after the transfer regardless of the time of day at which it was made.

These results are therefore entirely consistent with the idea that 5% CO_2 inhibits malate breakdown. Whilst this is believed to be the first report of the inhibition of a circadian rhythm by high ambient CO_2 concentrations, there have been a few studies on the effects of elevated CO_2 concentrations on plants performing CAM.

Exposing leaves of *Kalanchoë daigremontiana* to 5% CO_2 inhibits the deacidification phase of CAM (Nishida, 1977). This result could not be explained in terms of the closure of the stomata in response to 5% CO_2 because experiments were carried out with leaves from which the lower epidermis had been removed. More recently, Fisher and Kluge (1985) have investigated the effects of high CO_2 concentrations on the deacidification phase of CAM in *Kalanchoë tubiflora* and two species of *Sedum* by following the transfer of ^{14}C from malate into starch at various ambient CO_2 concentrations. Possible interference of high CO_2 concentrations with stomatal movements was eliminated by bisecting the leaves longitudinally before exposing them to enhanced CO_2 levels. At CO_2 concentrations of 2% and above the transfer of ^{14}C from malate into starch was inhibited. This inhibition was shown to be a direct effect of the increased CO_2 concentration on the malate removal process and not by inhibition of photosynthesis which was found to continue normally at the elevated CO_2 concentrations. Malate breakdown is inhibited by DCMU and stimulated by increasing light intensities (Nishida, 1982), the action spectrum for this stimulation being identical to that for photosynthesis (Barrow and Cockburn, 1982). Kluge and Fisher (1985) have suggested that the primary factor governing the rate of malate decarboxylation *in vivo* is light intensity. They suggest that light intensity controls the rate of photosynthesis which in turn determines the internal CO_2 concentration which ultimately controls the rate of malate decarboxylation. It must therefore be assumed that the enzymes responsible for malate breakdown require low internal CO_2 concentrations. High CO_2 concentrations have been shown to inhibit malic enzyme extracted from *Kalanchoë daigremontiana* (Walker, 1960). However, the CO_2 concentrations used in the study were much higher than those used in the present investigation.

It is also necessary to consider the possible influence of high ambient CO_2 concentrations on malate synthesis. The rate of malate synthesis in leaves maintained in 5% CO_2 may have been expected to be greater than in those kept in a stream of normal air. In the present investigation maximum malate levels occurred after leaves had been in normal air for 12 h, and 5% CO_2 for 16 h. Since no measurement was made of the malate status of leaves maintained in 5% CO_2 for between 4 and 16 h, however, the possibility that high CO_2 concentrations increased the rate of malate synthesis cannot be ruled out.

No evidence for increased malate synthesis in response to CO_2 concentrations of up to 6% was provided by the study of Kluge and Fisher (1985). In contrast, a few very early investigations indicated that night enrichment of the atmosphere with CO_2 increases the rate of malate synthesis and the final level accumulated. For example, Thomas and Ranson (1954) found a stimulation of malate synthesis in leaves of *Bryophyllum calycinum* in response to CO_2 concentrations within the range of 0 - 10 %, with an optimal effect between 2 and 5%. Within the range of 15 - 50% a reduction in the rate of synthesis and final level of malate accumulated was observed, and at concentrations above 50% dark acidification of the leaves was completely inhibited. These results were consistent with an *in vitro* study of the effects of high concentrations of CO_2 on the activity of PEPCase from *Kalanchoe* (Walker and Brown, 1957).

Similar findings to those of Thomas and Ranson (1954) were reported by Bonner and Bonner (1948). The latter investigation is of particular relevance to the present study since the effects of high CO_2 concentrations on malic acid synthesis

in a variety of CAM plants, one being *Bryophyllum fedtschenkoi*, was investigated. The amount of malate accumulated under different gas compositions was measured by comparing the concentration of malate in the leaves at the end of the photoperiod with that in leaves which had been maintained in darkness at 11°C in various concentrations of CO_2 for 48 h. All species investigated accumulated more malate in normal air than in CO_2-free air and more in 10% CO_2 than in normal air. The increase in the amount of malate produced in response to increased CO_2 concentrations, however, depended upon the species. For example, in *Bryophyllum crenatum* a large difference between the amount of malate accumulated in normal air and in 10% CO_2 was observed but in *Bryophyllum fedtschenkoi* this difference was very small. As discussed previously, leaves maintained in darkness and normal air for 48 h lose some of the malate which initially accumulates. Assuming that 10% CO_2 inhibits this loss, then the apparent increase in the amount of malate accumulated in 10% CO_2 with respect to that in normal air could be explained by the decrease which would occur in darkness and normal air.

Investigation of prolonged exposure of leaves of *Bryophyllum fedtschenkoi* to high and low temperature, darkness or 5% CO_2 has thus yielded substantial support for the hypothesis advanced by Wilkins (1984) for the generation and phase control of the rhythm of CO_2 exchange. These treatments inhibit the rhythm and set the phase in a manner which is entirely consistent with the malate status of the leaves. This finding suggests that malate synthesis and breakdown may be an integral part of the basic oscillator in *Bryophyllum*. In order to test further the validity of the hypothesis, a study of the induction of phase shifts in the rhythm persisting in continuous light was made by exposing leaves to high and low temperature, darkness and 5% CO_2 for a few hours at various points in the cycle. Where phase shifts were induced, the malate status of the leaves was examined to ascertain

whether or not this changed in a manner which would be predicted from the results of the prolonged exposure experiments.

Brief exposure of the leaves to 40°C, 2°C, darkness and 5% CO_2 were all found to be effective in inducing phase shifts in the rhythm otherwise operating in a stream of normal air and continuous illumination at 15°C. The magnitude of the phase shift induced depended upon the position in the cycle at which the treatment was applied. In general high temperature induced phase shifts in those positions in the cycle at which low temperature, darkness and 5% CO_2 were without effect on the phase and vice versa. The similarity of the effects of the latter three treatments on the rhythm extended to the direction of the phase shift induced.

In view of the large amount of information which was obtained from the investigation of phase control of the rhythm it is necessary to consider the effects of temperature, darkness and 5% CO_2 separately.

The magnitude of the phase shift induced by exposing leaves to 40°C for 4 h was dependent upon the position in the cycle at which the treatment was administered. Regardless of the time of day at which the high temperature stimulus ended, the next peak always occurred approximately 17 h later indicating that a brief exposure of the leaves to high temperature shifted the phase of the rhythm by forcing the basic oscillator to, and holding it at, a fixed phase point in the cycle from which it was released on restoring the temperature to 15°C.

Low temperature also appeared to induce phase shifts by forcing the basic oscillator to a fixed phase point in the cycle. The first peak following exposure of leaves to 2°C for 4 h always occurred approximately 7 h after the end of the treatment irrespective of the time at which it was administered.

The effects of short exposures to high and low temperatures on the phase of the rhythm were quite different with respect to the positions in the cycle at which they were effective or ineffective in inducing phase shifts. A high temperature treatment induced a maximum phase shift when it ended between the peaks of the

rhythm, and little or no phase shift when it ended at or near a peak in the rhythm. In contrast, a 2^0C stimulus, was most effective in inducing a phase shift when it ended a few hours after a peak and least effective when it ended in a trough.

The data presented in this thesis for the direction of phase shifts induced by high and low temperature are incomplete in that a whole cycle of CO_2 exchange was not scanned, but they nevertheless give a clear indication that high temperature advances the phase of the rhythm at those positions in the cycle at which low temperature induces phase delays and vice versa.

Phase control by high and low temperatures has been investigated in a wide variety of organisms. Some of these studies were outlined in the introduction. The results presented in this thesis indicate that in common with many other rhythms, the oscillator underlying the generation of the rhythm in *Bryophyllum* is sensitive to high temperature in those positions in the cycle at which it is insensitive to low temperature and vice versa. A large number of these investigations have indicated that low temperatures induce only phase delays, but it is clear that in *Bryophyllum*, phase advances and phase delays are produced in response to low temperature stimuli. The results in the present study are thus closely similar to those found for the K^+ uptake rhythm in *Lemna gibba* where the effects of high and low temperatures were also found to be opposite with respect to both the positions in the cycle at which they were effective in inducing phase shifts and in the direction of the phase shift induced (Kondo, 1983).

Several investigations have reported on the equivalence of darkness and low temperatures in inducing phase shifts (e.g. Wilkins, 1962b; Kondo, 1983; Lecharny *et al.*, 1985), and the results presented here conform with this generalisation. A short dark treatment was maximally effective in inducing phase shifts when it ended just after a peak in the rhythm and almost without effect when it ended in a trough. It will be recalled that similar findings were made for the effects of low temperature on the phase of the rhythm. However, a difference between the phase shifting effects of darkness and low temperature was also revealed in the present

study. The constant relationship which was found to exist between the time in the cycle at which a low temperature treatment ends and the occurrence of the next peak was not found for dark stimuli. There was a period of a few hours before and after the first peak during which a 4-h dark treatment induced a phase shift of approximately 6 h which appeared to be related to the duration of the treatment. Treatments ending a few hours before and after the second peak also induced phase shifts that seemed to be related to the duration of the treatment although these shifts were slightly larger (8 h). Only small phase shifts were induced by dark treatments ending before and after a trough in the rhythm and these did not appear to bear a relationship to the duration of the treatment, but rather to the position in the cycle at which the treatment ended.

A closely similar phase shifting pattern was produced when leaves were exposed to 5% CO_2 for 4 h at various positions in the cycle. The only apparent difference between the effects of darkness and 5% CO_2 on the phase was with respect to the magnitude of the phase shift induced at those positions in the cycle at which the shift appeared to be related to the duration of the treatment. A 5% CO_2 treatment ending at those positions induced a phase shift of approximately 4 - 6 h while a dark treatment shifted the phase by about 6 - 8 h.

The finding that 5% CO_2 was effective in inducing phase shifts is an important one since, as previously pointed out, it appears to be the first report of the effects of high concentrations of CO_2 on a circadian rhythm. It would be interesting to establish if such a treatment was effective in inducing phase shifts in other organisms, and if so whether or not it was equivalent to darkness with respect to the points in the cycle at which it was effective.

In addition to shifting the phase of the rhythm, high and low temperature and darkness also affected the subsequent period of oscillation in constant conditions. The alteration in the length of the period occurred regardless of whether the leaves

had been exposed to these treatments for a prolonged period or for only a few hours. Of particular interest was the finding that the period following exposure to 40°C was always longer than the period of the control rhythm while after a low temperature treatment the period was shorter than the control. Since the period was found to increase with increasing temperature these findings may indicate that the high and low temperature treatments leave some "after effect" on the temperature-compensating mechanism of the basic oscillator.

The effects of darkness on the subsequent period of the rhythm in the light are somewhat less clear. After prolonged exposure to darkness the period appeared to shorten. Contradictory results were, however, obtained for the effects of short-duration dark treatments on the period. In one series of experiments a 4-h exposure to darkness appeared to lengthen the subsequent period in continuous light while in later experiments a 4-h or 1-h dark treatment had no apparent effect on the period. At present it is impossible to suggest an explanation for these results.

The occurrence of transient instability in the period of circadian rhythms following a light or temperature signal is widespread in the plant and animal kingdoms (Bünning, 1973; Pittendrigh *et al.*, 1958). A detailed analysis of the lengths of successive periods following a dark or temperature treatment was not carried out in this investigation. It is clear, however, from the phase shifting data presented here, that the spectacular transients found in several other organisms do not occur in *Bryophyllum*. In the present study the difference between the periods of the control and treated leaves never exceeded 2 h and this difference appeared to remain more or less constant on successive peaks. In contrast, the first period in the leaf movement rhythm in *Phaseolus* following a temperature change was almost 5 h longer than the control rhythm but that of successive peaks was shorter (Leinweber, 1956). Similar findings have been reported in *Kalanchoë* (Oltmans, 1960).

The rhythm in *Bryophyllum* was not monitored for a sufficient length of time to establish how many transient cycles occurred before the steady-state was

reached. Such a study would require a prolongation of the time of each experiment to provide about 8 peaks, and it was felt that this would not be the best use of the time available for this investigation. The number of transient cycles which occur following a light or temperature stimulus appears to depend upon the organism under investigation. In *Euglena*(Bruce and Pittendrigh, 1958) only one transient cycle occurs before the final steady-state period is achieved while in *Drosophila* three transients occur following a light signal and two following a temperature stimulus (Pittendrigh *et al*., 1958). Pittendrigh *et al*. (1958) have concluded that the more complex the organism the greater the number of transients which occur following a light or temperature signal while the magnitude of the ultimate phase shift declines.

While the data on period instability in the present investigation are insufficient to provide the basis of an explanation they have raised several points which merit discussion.

It may be that the steady-state period is reached earlier after a dark treatment than a low temperature treatment. The reason for this tentative suggestion is that the fixed relationship between the end of a low temperature treatment and the time of occurrence of subsequent peaks appears to break down with time. There is evidence that by the time the third peak is reached after exposure to a low temperature treatment, the magnitude of the phase shift induced by treatments ending between 0200 h and 0600 h on the second day of the experiment is more related to the duration of the treatment than the time at which it ends (see Fig. 3.40.). This kind of relationship is observed in the first cycle following a dark and 5% CO_2 treatment. Since 5% CO_2 does not modify the period, the steady-state phase shift is attained immediately after such a treatment. It may be on reaching a steady-state period after low temperature or dark treatments, the phase shifting pattern would be identical to that produced by 5% CO_2. Further investigation would clearly be required, however, to test the validity of this view.

Pittendrigh *et al.* (1958) have proposed the existence of two oscillators to explain the phenomenon of transients. Briefly, this model assumes that an organism contains a master oscillator, the A oscillator, which is temperature insensitive but light sensitive. The A oscillator drives a B oscillator which is light insensitive and temperature sensitive. The phase of oscillator A is set immediately by a light signal and transient instabilities in the period reflect the re-entrainment of A by B; in the end a stable phase shift is observed. A temperature signal sets the phase of the B oscillator but A ultimately gains control of B, during which time transients are observed but only a small, if indeed any, phase shift results.

An evaluation of this hypothesis cannot be made on the basis of the data available on the *Bryophyllum* rhythm at present. The existence of coupled oscillators in this plant would seem highly unlikely in view of the finding by Wilkins (1960a and b) that transients did not occur in the rhythm of CO_2 output in CO_2- free air and darkness. Under these conditions the steady-state period was achieved immediately following a temperature or light stimulus. The difference in the results of Wilkins' study and those reported in the present investigation probably reflect the different way in which the period responds to phase shifting stimuli under different conditions.

Pittendrigh *et al.* (1958) do not discuss the possible nature of the A and B oscillators. The validity of their hypothesis is unlikely to be established until further information on the biochemical and molecular events underlying the generation of circadian rhythms is available.

Despite the large number of studies which have been carried out on the phase control of circadian rhythms by environmental parameters, very few have attempted to explain the mechanism of phase shifting in terms of what is known about the mechanism underlying the generation of the rhythm. A rather attractive hypothesis was advanced by Wilkins (1983) to explain the mechanism by which high temperature shifts the phase of the rhythm of CO_2 output in leaves of

Bryophyllum fedtschenkoi maintained in darkness and a CO_2- free air stream. The effects of short high temperature treatments on this rhythm were identical to those found in the present investigation but Wilkins (1983) was able to establish unequivocally the direction of the phase shifts induced by the high temperature stimuli at different points in the cycle. The hypothesis for the induction of phase shifts is wholly compatible with the mechanism proposed for the generation of the rhythm outlined earlier. It was postulated that "gates" exist in the tonoplast membrane which are closed in the dark but open in response to light and high temperature treatments allowing the leakage of malate from the vacuole into the cytoplasm where it inhibits PEPCase. Shortly after the end of these treatments the "gates" are thought to close and the rhythm begins by malate being pumped from the cytoplasm back into the vacuole. The magnitude of the phase shift depends upon how much malate leaks into the cytoplasm which in turn depends upon the relative concentration of malate in the cytoplasm and the vacuole, and on the duration of the treatment. In an earlier study Wilkins (1962b) reported that a maximum phase shift occurred in response to a 3-h high temperature treatment ending between the peaks in the rhythm. At this stage in the cycle PEPCase is at maximum activity therefore there can be little or no malate present in the cytoplasm; exposing leaves to high temperature opens the "gates" allowing malate to leak into the cytoplasm where it inhibits PEPCase. A 3-h treatment is assumed to be of sufficient duration to abolish completely the malate concentration gradient existing between the vacuole and the cytoplasm. The inability of a high temperature treatment to induce a phase shift when it ends across a peak in the rhythm is explained because at this stage PEPCase is inhibited anyway due to the high concentration of malate present in the cytoplasm; there is thus little or no concentration gradient across the tonoplast. The fixed phase point from which the rhythm begins at the end of a high temperature or light treatment is therefore represented by a state in which malate is evenly distributed between the vacuole and the cytoplasm. At the end of the treatment the rhythm always begins by malate

being pumped to the vacuole.

This hypothesis also accounts for the relationship between the position in the cycle at which the high temperature treatment ends and the direction of the phase shift induced. Phase advances are induced by treatments ending at times where the rate of CO_2 output is increasing. At this stage, the concentration of malate in the cytoplasm is thought to be increasing and the effect of a high temperature treatment would be to release the malate from the vacuole causing the concentration in the cytoplasm to increase more rapidly with the consequence that PEPCase is inhibited earlier than normal and a phase advance occurs. Phase delays occur in response to treatments ending a few hours after a peak in the rhythm when the rate of CO_2 output is decreasing and the activity of PEPCase is increasing. The effect of a high temperature treatment ending at these points in the cycle would be to allow malate which had been pumped into the vacuole to leak back out again restoring cytoplasmic malate to a level which had existed some hours earlier. At the end of the treatment the malate would have to be pumped into the vacuole resulting in a phase delay.

Wilkins is not alone in his view that phase shifts are induced by the alteration of ion gradients across membranes. The membrane model for the circadian clock (Njus *et al.* , 1973) accounts for the induction of phase shifts by light and temperature by proposing that these treatments alter ion gradients across the plasmalemma.

If Wilkins (1983) is correct in his proposal that "gates" in the tonoplast membrane open in response to light signals then his hypothesis for the induction of phase shifts by high temperature and light is clearly not applicable to the rhythm in light and normal air because in continuous light the "gates" must be permanently open. In view of the finding that prolonged exposure of the leaves to high temperature resulted in the malate status of the leaves being low while low temperature, darkness and 5% CO_2 led to a malate-rich leaf, a simple hypothesis to

explain the induction of phase shifts by stimuli of a few hours duration would be that these treatments alter the malate status of the leaves. This proposal, however, turned out to be incorrect; exposing leaves to high and low temperature, darkness and 5% CO_2 for 4 h, at a time in the cycle at which these treatments induced a large phase shift, did not significantly alter the malate status of the leaf. In retrospect, such a finding is hardly surprising since it was noted that during prolonged exposure of the leaves to low temperature, darkness and 5% CO_2 little change in the malate status of the leaves occurred during the first 4 h of the treatment. Similarly exposing malate-rich leaves to 40°C for 4 h was sufficient to reduce only partially the malate concentration in the cell sap. Whilst these findings clearly indicated that brief exposures to the above treatments do not alter the gross malate status of the leaves they do not rule out the possibility that localised changes in the malate concentration in a small cytoplasmic pool may occur. Before attempting to suggest how such a mechanism may operate it is necessary to consider the pattern of malate synthesis and breakdown in leaves maintained in continuous light and normal air at 15°C.

Under these conditions Nimmo *et al.*(1987) failed to detect periodic variations in the malate content of the leaves. Such a rhythm was, however, detected in the present investigation. The malate concentration of the expressed cell sap of individual leaves of *Bryophyllum* is somewhat variable making small oscillations difficult to detect. In order to minimise the effect of this variability, the number of leaves assayed at any one time in the present study was much greater than the sample sizes used by Nimmo *et al.* (1987) and it is probably this factor which accounts for the discrepancy between the results in the two studies. The light intensity was however, slightly lower in the present investigation than in that of Nimmo *et al.* (1987) and the higher fluence rate may have led to a damping in the malate oscillation, an effect which has been observed in *Kalanchoë daigremontiana* (Buchanan-Bollig, 1984).

The oscillation in the malate concentration of the cell sap was discernable for up to 72 h with a period of approximately 20 h. In contrast to the CO_2 exchange rhythm which persisted for many days, the malate oscillation had a much reduced amplitude and damped out so rapidly with time that the time of the third maximum was rather indistinct. Closely similar results have been reported in *Kalanchoë daigremontiana* (Buchanan- Bollig, 1984; Buchanan-Bollig and Smith, 1984).

On the basis of the hypothesis to explain the generation of the rhythm (Wilkins, 1984), the maxima in malate concentration would have been expected to coincide with the peaks in the CO_2 exchange rhythm since at these times PEPCase is thought to be inhibited by the large amounts of malate in the leaf cells. Minima in malate levels would have been expected to correspond with the occurrence of a trough in the rhythm since in the presence of very little malate, PEPCase would be expected to be maximally active. The results in the present investigation clearly do not support this hypothesis. The first maxima and minima in the malate oscillation occurred 4 - 6 h earlier than the first peak and trough in the CO_2 exchange rhythm. Because the periods of the two rhythms are different, the difference between the times of occurrence of the second maximum in malate and the peak in the CO_2 exchange rhythm was reduced to 2 - 3 h.

In contrast, Buchanan-Bollig and Smith (1984) found a correlation between the time of occurrence of the minima in malate concentration and the minima in CO_2 fixation in *Kalanchoë daigremontiana*. It is possible that this finding reflects more accurately the true state of affairs than the one revealed in the present investigation because malate determinations in the former study were made using leaf discs, so that in effect, the same leaf tissues were being used throughout the experiment. It is equally possible, however, that such a technique damaged the leaf cells resulting in an alteration in malate metabolism. Furthermore, CO_2 exchange was not monitored in the leaves which were used for the malate assays.

Since the CO_2 exchange rhythm and the pattern of malate accumulation in the cell sap were measured in the same leaves in the present study, there are strong grounds for believing that in leaves of *Bryophyllum fedtschenkoi*, the phases of the malate and the CO_2 exchange rhythms do not coincide.

It was noted that the first minima in the malate concentration of the leaves corresponded approximately with the time in the cycle at which high temperature had only a small effect on the phase while low temperature, darkness and 5% CO_2 induced relatively large phase shifts. High temperatures inhibit malate synthesis but allow malate breakdown to occur. Since the leaves were already in a malate-poor state no phase shift would have been expected. On the other hand, by inhibiting malate breakdown while allowing malate synthesis to occur, low temperature, darkness and 5% CO_2 may allow malate to build up in a localised pool in which PEPCase is located more rapidly than in untreated leaves. Thus, at the end of the treatment the leaves would have to remove this malate from the localised pool before a further period of fixation could occur, with the result that a phase shift is induced.

The amount of malate which would build up in the pool during these treatments might well be too small to allow detection by the present method against the overall malate status of the leaf. Good agreement was also found between the time of the maxima in malate content and the time in the cycle at which high temperature induced a large phase shift. Since PEPCase is presumably inhibited at this point anyway by the high concentrations of malate, it must be assumed that net malate breakdown occurs in the pool containing PEPCase. This would be achieved by high temperature enhancing the activity of malic enzyme and preventing further malate synthesis by inhibiting PEPCase. At the end of the treatment the localised pool in the leaves would be relatively malate-free and malate synthesis could begin earlier than in the untreated leaves resulting in a phase shift. Low temperature,

5% CO_2 and darkness would have been expected to have little effect on the phase at this time since these treatments lead to a malate-rich leaf and the leaves already have a malate-rich status. Scanning the cycle with dark and low temperature treatments did not begin until after the leaves had been in constant conditions for16 h so no phase shifting data are available at the time at which malate reached its first maximum, approximately 12 h after the start of the experiment. Exposure to 5% CO_2, however, had little effect on the phase at this time. At the time of the second maximum in malate content, however, low temperatures, darkness and 5% CO_2 did induce small phase shifts. This was also the time at which darkness and 5% CO_2 induced phase shifts which were apparently related to the duration of the treatment, rather than the time at which it ended. Thus, if this hypothesis is at all accurate in predicting the manner in which the above treatments shift the phase of the rhythm, then it is clearly a gross over-simplification of the actual mechanism. This hypothesis has attempted to explain only two of the characteristics of phase shifts observed here, namely the occurrence of phase shifts in certain positions in the cycle and not in others and the fact that the oscillator is sensitive to high temperature in those positions in the cycle in which it is insensitive to low temperature, darkness and 5% CO_2. A number of other points which have been raised by this investigation cannot be fully accounted for at present. These are as follows:

1. Why low temperature, darkness and 5% CO_2 are effective in inducing phase shifts at the same positions in the cycle, but the detailed pattern of phase shifting produced by low temperature is different from that produced by darkness and 5% CO_2.

2. Why high and low temperatures appear to drive the oscillator to, and hold it at, fixed phase points in the cycle.

3. Why the phase is advanced at certain positions in the cycle and delayed in

others and, in relation to this question, why the direction of the phase shift induced by a high temperature treatment ending a few hours before the first peak in the rhythm is the opposite to that induced by a similar treatment ending at the same time before the second peak.

No attempt will be made to speculate on how these points could be incorporated into the hypothesis outlined above; a few comments on their significance are, however, justified.

Firstly, the reason for the apparent difference in the phase shifting pattern induced by low temperature, darkness and 5% CO_2 may lie in the precise way in which these treatments bring about an inhibition in the rhythm. Low temperature may inhibit malate breakdown simply because malic enzyme cannot operate below a certain temperature, while CO_2 may compete with malate for malic enzyme. The effect of darkness may also be attributed to the induction of high internal concentrations of CO_2. A further possibility, however, with respect to the way in which dark treatments bring about phase shifts, must be considered. If Wilkins is correct in his view about "gates" in the tonoplast, then the effect of a dark treatment would be to close these "gates" thereby allowing malate to be removed to the vacuole.

High and low temperatures appear to force the basic oscillator to fixed phase points. It takes 2 - 3 h longer for the first peak of the rhythm to occur after a short exposure to these treatments than it does after a prolonged exposure. This finding may indicate that the 4-h treatments do not result in the oscillator achieving the same phase point as it does after prolonged treatments. It will be recalled that the latter treatments alter the gross malate status of the leaves while the former do not. If the size of the phase shift is related to the change in malate levels either in the cell as a whole, or in a localised pool, then the magnitude of the phase shift induced by high or low temperature treatments would be expected to depend upon the duration of the treatment up to a certain threshold time. Time did not permit such

thresholds to be determined in this study, but the data available may indicate that a 4-h treatment might not be a fully saturating one.

The results presented in this thesis did not allow the direction of the phase shifts induced by various treatments to be conclusively established. It would, therefore, be premature to attempt to explain this point in terms of the malate status of the leaves. It was somewhat surprising to find that a high temperature ending before the first peak of the rhythm shifted the phase in the opposite direction to a similar treatment ending at the same time before the second peak. This finding may indicate that the malate status of leaves a few hours before the first peak of the rhythm is different to that which exists a few hours before the second peak.

An adequate explanation to account for all the features of phase shifting reported here must necessarily await a fuller understanding of the mechanism underlying the generation of the rhythm. The results obtained for the pattern of malate accumulation in light and normal air do indeed raise doubts about the validity of the hypothesis to account for the generation of the rhythm under these conditions, but a recent study by Nimmo *et al.* (1987) has provided some support for the hypothesis on the generation of the rhythm in CO_2-free air and darkness (Bollig and Wilkins, 1979; Wilkins, 1983).

A key enzyme to feature in both these hypotheses, PEPCase, plays an important role in the CAM pathway and there is overwhelming evidence to suggest that this enzyme is regulated by the periodic accumulation of malate in the cytoplasm (e.g. Kluge and Ting, 1978; Osmond and Holtum, 1981). The kinetic properties of PEPcase from a number of CAM plants have been shown to vary diurnally (see introduction). In *Bryophyllum fedtschenkoi*, diurnal changes in the form of PEPCase are associated with a rhythm in the phosphorylation state of this enzyme (Nimmo *et al.*, 1986). The night form of the enzyme is phosphorylated on one or more serine residues and is characterised by having a high K_i for malate. The day form of PEPcase is dephosphorylated and is ten times more sensitive to inhibition

by malate.

Persistent rhythms in the phophorylation state of PEPCase from *Bryophyllum* have recently been found to occur under constant conditions (Nimmo *et al.*, 1987). In CO_2- free air and darkness, the dephosphorylated form, having a low K_i for malate, was found to occur at times when peaks in the CO_2 output rhythm occurred, and the phosphorylated form, which is less sensitive to inhibition by malate, occurred at times when the rate of CO_2 output was decreasing. Although no rhythm in the specific activity of PEPCase was observed, Nimmo *et al.* (1987) suggest that the phosphorylated form of the enzyme is more active *in vivo* than the malate sensitive dephosphorylated form.

The malate status of the leaves maintained in CO_2-free air and constant darkness was also determined and was not found to vary but rather to remain at a constantly low level (Nimmo *et al.*, 1987). Since this rhythm has been attributed to the periodic accumulation of malate in the cytoplasm followed by its removal to the vacuole no oscillation in malate would have been expected but rather a step-wise daily increase as it gradually accumulated in the vacuole. No such step-wise accumulation of malate was observed during the first two days in darkness, but it is possible that the amount of malate synthesised in each 24-h cycle is relatively small in a CO_2-free air stream and this, together with the inherent variability of malate levels in different leaves, may have obscured any increase which might have occurred. The present investigation has revealed that malate breakdown can occur for a short time in darkness and it is possible that a futile cycle of malate synthesis and breakdown also occurs under such conditions. In CO_2- free air, CO_2 is obviously limiting, and CO_2 derived from malate decarboxylation may be immediately refixed via PEPCase to produce malate. This cycle would continue until eventually malate built up to a level which inhibited PEPCase. Such an explanation implies that the rate of malate synthesis must be slightly faster than the rate of malate breakdown.

An increase in the malate content of the leaves may not therefore be observed during the first 48 h in these conditions but might occur after several days. The hypothesis of Wilkins (1984) cannot therefore be discarded on the basis of these findings.

A rhythm in the kinetic properties of PEPCase also persisted in leaves maintained in continuous light and normal air (Nimmo *et al.*, 1987). This rhythm was, however, found to be out of phase with the rhythm of CO_2 exchange. The phosphorylated form of the enzyme, having a high K_i for malate, occurred slightly later than the times of maximum CO_2 uptake. A rather striking correlation exists between this phosphorylation rhythm and the malate rhythm observed in the present investigation. Conversion of the dephosphorylated form of the enzyme to the phosphorylated form coincided almost exactly with the time at which leaves had attained their maximum malate levels, and the time at which dephosphorylation occurred coincided with the time at which the malate concentration in the cell sap had reached its lowest level. Furthermore, a rapid damping of the phosphorylation rhythm occurred in leaves maintained in continuous light for several days.

The kinetic properties of PEPCase from *Kalanchoë daigremontiana* have also been found to vary in continuous light. In contrast to the rhythm in *Bryophyllum*, however, this rhythm only persisted for about 18 h (Buchanan-Bollig and Smith, 1984).

The results of the study by Nimmo *et al.* (1987) and those in the present investigation provide some evidence to suggest that PEPCase is regulated by malate levels in leaves maintained in constant condtions. To what extent the rhythms in the gross malate content of the leaves and the phosphorylation state of PEPCase contribute to the generation of the rhythm in CO_2 exchange in continuous light is unclear at present. When leaves were transferred to continuous light at the end of the normal night, a marked rhythm of CO_2 exchange was detected but no

interconversion of the phosphorylation state of PEPCase occurred; the enzyme persisted in the dephosphorylated state throughout the experiment. During the first 8 h in the light the malate content of the leaves declined, and thereafter remained relatively low. It would be interesting to establish whether the apparent absence of a rhythm in gross malate content was due to the small sample size, the high light intensity used, or to a true inhibition of the rhythm.

A number of findings therefore raise serious doubts that the rhythm of CO_2 exchange in light and normal air is a result of an underlying rhythm in PEPCase activity brought about by the periodic synthesis and breakdown of the gross malate content of the leaf. In summary, these findings are as follows:

1. The rhythms in the phosphorylation state of PEPCase (Nimmo *et al.*, 1987) and in the gross malate content of the leaves are out of phase with the rhythm of CO_2 exchange.

2. The rhythms in the phosphorylation state of PEPCase and in the gross malate content persist for only a few days under constant conditions, while the CO_2 exchange rhythm continues for many days.

3. Under certain conditions a rhythm of CO_2 exchange occurs in the apparent absence of rhythms in the phosphorylation state of PEPCase and the gross malate content of the leaves.

Nimmo *et al.* (1987) have suggested that in continuous light the rhythm of CO_2 exchange may reflect a rhythm in both PEPCase and ribulose bisphosphate carboxylase (RuBP) activity, and there have indeed been demonstrations that CAM plants can fix CO_2 directly via both the C3 and C4 pathways. (Winter, 1980b; Ritz, Kluge and Veith, 1986).

In an attempt to establish the relative contribution of PEPCase and RuBP activity in the generation of the rhythm in CO_2 exchange, Buchanan-Bollig *et al.* (1984)

have followed the pathway of $^{14}CO_2$ fixation in *Kalanchoë daigremontiana* maintained in normal air and continuous illumination. Whilst both the C4 and C3 pathways were found to operate under these conditions no rhythmic activity in the C3 pathway was detected. At moderate irradiances (10 - 60 W m^{-2}), however, incorporation of $^{14}CO_2$ into C4 products showed a marked circadian rhythm, and determination of the intramolecular label distribution indicated that this rhythm was not due to an oscillation in the availability of photosynthetically pre-labelled C3 skeletons but to a true rhythm in the C4 pathway. At high irradiances this rhythm damped out in parallel with the rhythm of CO_2 exchange, and that of malate accumulation (Buchanan-Bollig and Smith, 1984). All three rhythms were completely abolished at 120 W m^{-2}; at this light intensity CO_2 was fixed at a constant low rate, both C3 and C4 products were labelled and the malate concentration remained relatively low. Pulse-chase experiments revealed that even at times of maximum $^{14}CO_2$ uptake, the activity of the decarboxylating enzymes remained high, indicating that the rhythm was not caused by rhythmic activity in the pathway of malate breakdown. Furthermore, the turnover rate of malate in continuous light was found to be much higher than in a normal light-dark cycle. These authors concluded that the rhythm of CO_2 exchange in light and normal air reflected a rhythm in PEPCase activity, but in continuous light the storage capacity of the vacuole was impaired, a view which is entirely consistent with Wilkins' hypothesis (1984). Unfortunately rhythmic incorporation of $^{14}CO_2$ was followed only during the first 60 h in continuous light when a rhythm in the malate content of the leaves was still detectable. It would be interesting to establish whether or not the rhythm continued even after the oscillation in malate had damped out.

The rhythm of CO_2 compensation persisting in leaves of *Bryophyllum fedtschenkoi* in continuous light is also thought to arise from a rhythm in the

activity of PEPCase (Jones and Mansfield, 1973). Rhythmic incorporation of $^{14}CO_2$ was observed when leaves were labelled at high and low light intensities and in the dark. Further support for this conclusion was obtained from measurements made on the total titratable acidity of the shoots maintained in a light-dark cycle, and in continuous light for 24 h. At the onset of the light period both samples showed a marked reduction in their acid content but this increased again in leaves exposed to darkness and to a lesser extent in leaves maintained in the light. It would be interesting to establish if this rhythm persisted for more than one cycle and if so whether or not its phase bore any relationship to that of the CO_2 compensation rhythm. The $^{14}CO_2$ studies in this investigation were carried out in leaves from which the epidermis had been removed thus eliminating the possibility that the rhythm was due to an oscillation in stomatal movement.

The possibility that the rhythm reported in this thesis reflects a rhythm in stomatal movements was not investigated but appears to be extremely remote in view of a finding in *Kalanchoë diagremontiana* (Buchanan-Bollig and Smith, 1984). Simultaneous monitoring of CO_2 exchange and transpiration rates in that species in continuous light and normal air revealed a marked circadian rhythm of CO_2 exchange while transpiration persisted at a constantly high rate indicating that the stomata were permanently open under these conditions.

Examination of the stomatal movements of *Bryophyllum fedtschenkoi* was attempted using light and infra-red microscopy with an infra-red camera to visualise the images. The stomata in this species are deeply sunk beneath the waxy surface of the leaf and changes in the aperture could not therefore be seen. It was clear that the wax layers would need to be removed before such a study could be made and the effect of this treatment on the stomata might be significant. Time did not permit this line of investigation to be pursued.

The results presented in this thesis have not provided conclusive evidence

either for or against the hypothesis advanced by Wilkins (1984) for the generation of the rhythm in leaves of *Bryophyllum fedtschenkoi* maintained in light and normal air. They have, however, firmly established that prolonged exposure of the leaves to high temperature controls the phase of the rhythm by holding the oscillator at a fixed phase point characterised by a leaf which contains relatively low levels of malate. Prolonged periods of low temperature, darkness and 5% CO_2, on the other hand exert their effects on the phase by forcing the basic oscillator to a fixed phase point in the cycle characterised by a malate-rich leaf. What remains uncertain, however, is the way in which short exposures of the leaves to these treatments shift the phase of the rhythm. Such phase shifts are clearly not characterised or associated with an alteration in the gross malate status of the leaves, but it is possible that brief exposure of the leaves to these phase shifting stimuli induces an alteration in the malate level in a localised pool in which PEPCase is present. These localised changes might be quite large but nevertheless undetectable against the overall malate content of the leaf cells with the analytical procedure employed. If the rhythm in CO_2 exchange reflects an underlying oscillation in PEPCase activity brought about by localised periodic accumulation of malate and, if as suggested by Wilkins (1983) and Buchanan-Bollig (1984), the storage capacity of the vacuole is disrupted in continuous light, then PEPCase must be isolated from the main site of malate accumulation since malate builds up at a time when the rate of CO_2 uptake is increasing, which presumably means that the activity of PEPCase is also increasing.

One of the main points against the idea that a rhythm in malate synthesis underlies the CO_2 exchange rhythm is the fact that the former oscillation damps out much more rapidly than the latter. This finding does not, however, demolish the hypothesis since rhythmic synthesis and breakdown of malate may continue to occur in a localised pool even after the rhythm in the gross malate content of the leaves has damped out. The hypothesis for the generation of the rhythm (Wilkins,

1984), and the mechanism which was suggested for phase shifting in the present investigation, clearly depend upon PEPCase being located in a compartment within the cytoplasm. It was pointed out in the introduction that there is controversy over the intracellular location of PEPCase; the majority of reports claim that it is located in the cytoplasm, while a few have suggested that it may be located in the chloroplast. It is now critically important that the intracellular location of the enzymes involved in the CAM pathway be unequivocally established. Such information on whether PEPCase is located in a cytoplasmic compartment or in an organelle will be of value in assessing the hypothesis proposed here for phase shift induction, and form the basis on which to test the hypothesis further. Conclusive proof of the validity of the hypothesis would require techniques to measure the level of malate in the different organelles and compartments within the cell. Measurement of the level of malate in the cytoplasm and vacuole at various times throughout the day could also provide information on the validity of the mechanisms proposed for the generation of the rhythm in CO_2-free air and darkness (Bollig and Wilkins, 1979) and for phase shift induction in this rhythm (Wilkins, 1983).

Further steps towards the understanding of the mechanism underlying the generation of the circadian rhythm of CO_2 exchange, and the mechanism of phase control need not await the unequivocal localisation of the CAM enzymes, or the technology which will enable measurements of malate to be made in cell organelles or cytoplasmic compartments. There are many other aspects of the rhythm which remain to be investigated and which will undoubtedly lead to a better understanding of the generation of the rhythm.

Investigation in this laboratory has focused mainly on the regulation of PEPCase by malate and its role in the generation of the rhythm. It is possible that malic enzyme, the enzyme responsible for malate breakdown, may also play a part. No evidence of a rhythm in the activity of the malate degradation pathway was

found in *Kalanchoë diagremontiana* (Buchanan-Bollig *et al.*, 1984), and in a preliminary study of malic enzyme in this laboratory no change in its kinetic properties was detected when the enzyme was extracted from leaves at opposite phases in the circadian cycle (Forrest, unpublished data). Other studies have claimed, however, that malic enzyme and PEPCase activities fluctuate out of phase with each other (see Kluge and Ting, 1978). There have, on the whole been very few studies carried out on malic enzyme and further investigation is fully justified in view of the conflicting results which are already to hand.

One of the major difficulties in relating the oscillation in malate levels to the CO_2 exchange rhythm in continuous light was the variability of the malate content of individual leaves. Since the amplitude of the malate oscillation was very small, this variability may have obscured small periodic fluctuations. In a normal light-dark cycle, however, marked oscillation in malate occurs (Nimmo *et al.*, 1984). It would, therefore, be interesting to establish the relationship between the malate rhythm and the CO_2 exchange rhythm in leaves which were being entrained to various periodicities by light-dark cycles. If the period of the malate rhythm altered in response to changing light-dark cycles in a similar manner to the CO_2 exchange rhythm, it would provide good evidence that the two rhythms are intimately associated and that oscillations in malate concentration are an integral part of the basic oscillator in *Bryophyllum*.

No hypothesis for the generation of circadian rhythms has yet been able to account for the length of the period, or the fact that it is temperature-compensated. Stimuli which alter the length of circadian periods are important probes in the study of these rhythms, since they undoubtedly act on the basic oscillator rather than on the overt rhythm. Wilkins (1984) has suggested that the period of the CO_2 exchange rhythm exhibited by leaves of *Bryophyllum* in light and normal air may be under the control of light intensity. Higher light intensities would lead to higher rates of photosynthesis which might, in turn, speed up the rate of the

deacidification phase of CAM as the end products from malate breakdown would be immediately consumed in photosynthesis. The period of the CO_2 exchange rhythm in *Kalanchoë daigremontiana* is clearly affected by the light intensity (Buchanan-Bollig, 1984). Increasing the fluence rate between 5 and 60 W m^{-2} decreased the length of the free-running period. Indeed, in the present investigation a slight reduction in the length of the free-running period was observed when a fluorescent tube giving a slightly higher light intensity replaced one which had failed. However, reducing the light intensity from 18 to 4.7 J m^{-2} s^{-1} was not found to alter the period of the CO_2 compensation rhythm in *Bryophyllum fedtschenkoi* (Jones, 1973). Further investigation of the role of light intensity in controlling the periods of circadian rhythms in this species is clearly required.

If rhythmic changes in the level of malate in the cells is an integral part of the basic circadian oscillator in leaves of *Bryophyllum fedtschenkoi* then clearly this would dispute the idea that a basic oscillator common to all organisms underlies the generation of circadian rhythms. At the present stage in this investigation, however, the possibility that the rhythm of CO_2 exchange is a manifestation of a more fundamental basic oscillator cannot be dismissed.

Progress towards elucidating the mechanism underlying circadian rhythms is slow, but their generation and control by environmental parameters is so complex that a thorough investigation of the physiological characteristics of the rhythms must necessarily precede biochemical and molecular studies. There is little doubt, however, that it is now appropriate to attempt to investigate the rhythm in *Bryophyllum fedtschenkoi* at the sub-cellular level, especially to address the question of the localisation and compartmentation of the reactants and products involved in the CAM pathway, and the movement of the various substances between compartments. Such studies will not be easy, but they might yield a real insight into the generation and control of a circadian oscillation in at least one organism.

5. APPENDICES

APPENDIX 1.

CALIBRATION OF THE ADC INFRA-RED GAS ANALYSER.

One division on the scale of the IRGA = 1ppm CO_2.

Because at 1ppm, 10^6 litres of air contains 1 litre of CO_2, 1 litre of air will contain 1 microlitre of CO_2

Every hour, 3 litres of air flow through the IRGA, therefore 3 microlitres of CO_2 must flow through in each hour for each scale division on the IRGA.

The results are expressed in $\mu g\ CO_2\ h^{-1}\ g\ (\text{fresh weight})^{-1}$, therefore the weight of 3 microlitres of CO_2 must be found.

One mole of any gas at standard temperature and pressure (STP) occupies 22.4 litres

One mole of any gas at 25°C occupies 24.4 litres

Gram molecular weight of CO_2 = 44

24.4 microlitres is will weigh 44 µg at 25°C

3 microlitres will weigh $\frac{3 \text{ microlitres} \times 44 \text{ g}}{24.4 \text{ microlitres}} = 5.4098$

One division on the IRGA scale = 5.4098 $\mu g\ CO_2\ h^{-1}$

APPENDIX 2

The Effects of Various Environmental Parameters and Changes in Environmetal Parameters on the Phase and Period of the Rhythm of CO_2 Exchange

In all tables where data for the lengths of the period of the rhythm are given these figures refer to the mean period length in hours ± the S.E. of the mean. The number in brackets refer to the number of individual readings contributing to the mean. When t values are given - N.S. = Not significant, * = significant at 0.05 level of probability, ** significant at 0.01 level of probability.

TABLE A

THE EFFECTS OF TEMPERATURE ON THE PERIOD

TEMPERATURE ^{o}C	PERIOD ± S.E.
10	15.7 ± 0.35 (59)
12.5	16.8 ± 0.31 (21)
15	18.2 ± 0.16 (70)
18	18.4 ± 0.27 (24)
20	19.7 ± 0.17 (50)
25	20.5 ± 0.38 (36)
28	20.9 ± 0.51 (23)
30	22.3 ± 0.33 (18)

TABLE B

SUCCESSIVE PERIOD LENGTHS AT VARIOUS CONSTANT AMBIENT TEMPERATURES

	PERIOD NUMBER			
TEMPERATURE IN °C	1	2	3	4
10	18.0 ± 0.45 (9)	16.2 ± 0.39 (9)	16.4 ± 0.56 (9)	15.3 ± 0.59 (9)
12.5	17.5 ± 0.46 (8)	16.7 ± 0.46 (8)	16.1 ± 0.78 (5)	
15	17.9 ± 0.33 (12)	17.9 ± 0.19 (12)	18.3 ± 0.48 (12)	18.1± 0.27 (12)
18	17.2 ± 0.21 (6)	18.5 ± 0.26 (6)	18.9 ± 0.36 (6)	19.0 ± 0.37 (6)
20	19.4 ± 0.61 (7)	19.1 ± 0.18 (7)	20.1 ± 0.30 (7)	19.8 ± 0.37 (7)
25	17.2 ± 0.30 (5)	21.7 ± 0.30 (5)	20.3 ± 0.29 (5)	20.4 ± 0.29 (5)
28	21.4 ± 0.80 (5)	22.4 ± 1.60 (5)	21.5 ± 0.41 (5)	19.7 ± 0.87 (5)
30	22.1 ± 0.59 (9)	22.5 ± 0.36 (9)		

	PERIOD NUMBER			
TEMPERATURE IN °C	5	6	7	8
10	14.2 ± 0.17 (9)	13.7 ± 0.25 (6)	16.2 ± 0.17 (6)	15.3 ± 1.1 (2)
15	18.5 ± 0.48 (10)	18.4 ± 0.44 (6)	18.1 ± 0.40 (6)	
20	20.0 ± 0.42 (9)	20.1 ± 0.52 (5)		
25	22.0 ± 0.34 (6)	21.4 ± 0.80 (6)		
28	19.6 ± 0.67 (3)			

TABLE C

VALUES OF t OBTAINED FROM A COMPARISON OF THE INDIVIDUAL PERIODS PRESENTED IN TABLE B AT VARIOUS TEMPERATURES

	PERIOD NUMBERS COMPARED						
TEMP ^{o}C	1&2	1&3	1&4	1&5	1&6	1&7	1&8
10	2.284**	1.840	3.880**	6.461**	9.398**	4.494**	4.634**
12.5	1.980	1.550	0.533				
15	0.100	1.234	0.237	1.397	1.713	0.107	
18	2.200*	2.846*	2.946*				
20	0.340	1.06	1.519	0.870	0.966		
25	10.607**	8.845**	7.650**	7.770**			
28	0.660	0.160	1.600	2.000			
30	1.137						

TABLE C CONTINUED

PERIOD NUMBERS COMPARED

TEMP °C	2&3	2&4	2&5	2&6	2&7	2&8	3&4
15	0.234	0.241	1.432	1.644	0.207	0.109	1.239
18	0.859	1.338					0.756
25	1.912	3.100*	0.486	0.800			1.213
28	1.480	1.544	1.717				

PERIOD NUMBERS COMPARED

TEMP °C	3&5	3&6	3&7	3&8	4&5	4&6	4&7
15	0.871	0.862	0.401	0.321	0.800	1.069	0.173
25	1.792				2.600*	1.438	

PERIOD NUMBERS COMPARED

TEMP °C	5&6	5&7	6&7	6&8	7&8
10			8.030**	1.412**	
15	0.354	0.732	0.80		

TABLE D

VALUES OF t OBTAINED FROM A COMPARISON OF THE EFFECTS OF VARIOUS CONSTANT AMBIENT TEMPERATURES ON THE PHASE OF THE RHYTHM

TEMPERATURES (^{o}C) COMPARED	t	S
15 & 18	1.250	N.S.
15 & 20	2.831	*
15 & 25	3.081	*
15 & 10	1.151	N.S.
15 & 12.5	0.821	N.S.
18 & 20	4.253	**
18 & 25	4.511	**
18 &10	2.213	N.S.
18 &12.5	0.536	N.S.
20 & 25	0.267	N.S.
20 & 10	1.137	N.S.
20 & 12.5	4.045	**
25 & 10	1.337	N.S.
25 & 12.5	4.324	**
10 & 12.5	1.897	N.S.

Data refer to Table 2., page 69.

TABLE E

COMPARISON OF THE PERIOD IN LEAVES TREATED IN VARIOUS WAYS WITH THAT IN CONTROL LEAVES KEPT IN CONTINUOUS LIGHT AND NORMAL AIR AT 15°C

	PERIODIN HOURS			
TREATMENT	TREATED	CONTROL	t	S
Prolonged 40°C	19.4 ± 0.17 (39)	18.5 ± 0.13 (113)	2.394	*
4 h 40°C	19.6 ± 0.12 (157)	18.5 ± 0.15 (113)	2.471	*
+4 h 40°C	19.2 ± 0.30 (59)	18.3 ± 0.17 (28)	2.649	*
1 h 40°C	19.1 ± 0.12 (59)	18.3 ± 0.17 (28)	2.284	*
Prolonged 2°C	17.8 ± 0.25 (46)	18.9 ± 0.15 (43)	5.840	**
4 h 2°C	17.6 ± 0.15 (156)	18.4 ± 0.16 (103)	2.170	*
+4 h 2°C	17.2 ± 0.24 (22)	18.3 ± 0.18 (29)	3.037	**
1 h 2°C	17.4 ± 0.18 (21)	18.3 ± 0.18 (29)	2.544	*
Prolonged dark	17.4 ± 0.20 (21)	19.3 ± 0.31 (39)	5.567	**
4 h dark	19.40 ± 0.21 (132)	18.4 ± 0.19 (98)	3.102	**
+4 h dark	17.7 ± 0.41 (15)	17.6 ± 0.52 (25)	0.316	N.S.
1 h dark	17.9 ± 0.39 (15)	17.6 ± 0.52 (25)	0.901	N.S.
prolonged CO_2	19.2 ± 0.61 (18)	18.8 ± 0.32 (13)	0.480	N.S.
4 h 5% carbon dioxide	18.8 ± 0.21 (143)	17.9 ± 0.21 (117)	0.675	N.S.
+4 h 5% carbon dioxide	17.7 ± 0.38 (11)	17.9 ± 0.21 (16)	0.875	N.S.
1 h 5% carbon dioxide	17.6 ± 0.30 (11)	17.2 ± 0.21 (16)	0.364	N.S.
2°C + 8 h 40°C + 15°C	19.4 ± 0.50 (7)	18.1 ± 0.20 (8)	2.348	*
2°C + 4 h 40°C + 15°C	17.9 ± 0.30 (9)	16.3 ± 0.39 (5)	2.592	*

+Data obtained during experiments to establish direction of phase shift. Underlined control data were for leaves maintained at 2°C before being transferred to 15°C.

APPENDIX 3.

The Concentration of Malate (mM) and pH of the Cell Sap of Leaves Subjected to Various Treatments.

Data refer to the mean concentration of malate and pH of the cell sap of leaves ± the S.E. of the mean values

TABLE F

MALATE CONCENTRATION OF THE CELL SAP OF LEAVES EXPOSED TO 2°C, 40°C, DARKNESS AND 5% CO_2

DURATION OF	TREATMENT			
TREATMENT	2°C	40°C	DARK	5% CO_2
0	15.0 ± 3.09	18.9 ± 4.10	16.9 ± 3.24	21.2 ± 2.10
2			25.7 ± 2.19	
4	18.5 ± 3.28	15.6 ± 3.20	22.3 ± 2.38	20.4 ± 2.46
6	24.2 ± 2.75		32.9 ± 1.94	
8	32.1 ± 3.20		34.6 ± 2.31	
10	30.8 ± 3.56		41.3 ± 2.79	
12	33.2 ± 4.30		46.0 ± 3.71	
14			53.2 ± 5.50	
16	41.0 ± 3.06	7.0 ± 2.20	41.0 ± 2.76	42.1 ± 2.10
24	47.3 ± 3.49	11.3 ± 0.80	39.0 ± 2.78	40.0 ± 2.10
40	50.2 ± 3.78	13.4 ± 2.90	34.7 ± 2.69	47.3 ± 5.36
48	45.4 ± 3.67	9.3 ± 3.10	34.2 ± 1.80	36.7 ± 2.54
68	48.3 ± 3.67			50.8 ± 3.49
72	44.9 ± 2.75	7.0 ± 3.30	33.9 ± 3.40	

Each reading represents the mean value of 6 individual readings

TABLE G

pH OF THE CELL SAP IN LEAVES EXPOSED TO 2°C AND DARKNESS

DURATION OF TREATMENT	TREATMENT	
	2°C	DARK
0	5.6 ± 0.35	5.9 ± 0.37
2		5.6 ± 0.18
4	5.1 ± 0.13	5.8 ± 0.16
6	5.3 ± 0.20	5.3 ± 0.18
8	5.0 ± 0.21	5.2 ± 0.13
10	4.9 ± 0.10	5.1 ± 0.17
12	4.9 ± 0.09	5.0 ± 0.13
14		4.9 ± 0.19
16	4.2 ± 0.14	4.8 ± 0.15
24	3.9 ± 0.14	4.8 ± 0.12
40	4.2 ± 0.12	4.9 ± 0.11
48	4.1 ± 0.14	4.9 ± 0.14
70	4.2 ± 0.11	5.0 ± 0.18

Each reading represents the mean value of 6 individual readings

TABLE H

CONCENTRATION OF MALATE AND pH IN THE EXTRACTED CELL SAP OF LEAVES EXPOSED TO 2°C FOR 44 h AND THEN 40°C FOR VARIOUS DURATIONS

TIME AT 40°C AFTER 44 H AT 2°C	[MALATE IN CELL SAP (mM)]	pH
0	42.6 ± 1.19	4.2 ± 0.23
1	40.2 ± 1.60	4.3 ± 0.25
2	44.6 ± 4.40	4.4 ± 0.21
4	33.9 ± 3.68	4.4 ± 0.20
8	25.7 ± 4.44	4.9 ± 0.15
12	27.1 ± 2.87	5.2 ± 0.25
16	24.4 ± 0.92	5.2 ± 0.12
20	27.3 ± 1.60	5.3 ± 0.12
24	22.3 ± 5.00	5.3 ± 0.14
28	23.3 ± 2.10	5.3 ± 0.25

Each reading represents the mean value of 4 individual readings.

TABLE I

MALATE CONCENTRATION AND pH OF THE CELL SAP OF LEAVES HELD IN LIGHT AND NORMAL AIR AT 15°C FOR VARIOUS DURATIONS

TIME AT 15°C	mM MALATE IN CELL SAP	pH
0	25.3 ± 2.25	5.3 ± 0.12
4	36.1± 3.35	5.0 ± 0.05
8	43.7 ±2.77	4.9 ± 0.09
12	55.5 ± 2.84	4.7 ± 0.06
16	43.0 ± 2.04	4.9 ± 0.08
20	34.3 ± 2.00	4.9 ± 0.09
24	38.7 ± 1.70	5.1 ± 0.06
28	44.7 ±1.50	4.9 ± 0.03
32	43.5 ±1.27	4.9 ± 0.06
36	41.7 ±1.74	4.9 ± 0.04
40	35.4 ± 1.71	5.1 ± 0.06
44	35.9 ± 1.38	5.2 ± 0.04
48	34.0 ± 1.97	5.0 ± 0.28
52	28.4 ± 2.04	5.1 ± 0.06
56	32.5 ± 3.28	5.0 ± 0.07
60	34.4 ± 2.24	5.3 ± 0.03
64	23.4 ± 1.20	5.4 ± 0.04
70	28.6 ±1.64	5.1 ± 0.08

Data relate to Fig. 3.46B. Each reading represents the mean value of 12 invidual readings.

TABLE K

CONCENTRATION OF MALATE IN THE EXTRACTED CELL SAP OF LEAVES HELD IN LIGHT AND NORMAL AIR AT 15°C FOR VARIOUS DURATIONS

TIME AT 15°C	mM MALATE IN CELL SAP	TIME AT 15°C	mM MALATE IN CELL SAP
0	26.3 ± 2.30	38	40.0 ± 6.42
2	13.9 ± 2.40	40	41.4 ± 3.41
4	17.0 ± 2.67	42	40.0 ± 6.59
6	23.8 ± 3.66	44	26.5 ± 2.10
8	28.6 ± 3.16	46	34.2 ± 3.68
10	41.9 ± 5.83	48	36.4 ± 4.36
12	46.3 ± 8.25	50	30.7 ± 6.93
14	61.7 ± 10.73	52	26.8 ± 2.78
16	57.8 ± 5.01	54	32.4 ± 6.57
18	57.8 ± 6.30	56	30.9 ± 3.06
20	26.4 ± 4.60	58	39.8 ± 4.86
22	35.2 ± 5.25	60	38.0 ± 7.92
24	21.6 ± 3.86	62	36.1 ± 6.29
26	24.9 ± 2.87	64	37.2 ± 7.49
28	37.2 ± 3.14	66	28.9 ± 4.44
30	34.6 ± 3.70	68	25.2 ± 5.82
32	33.5 ± 3.50	70	21.5 ± 2.78
34	42.8 ± 3.47		

Data relate to Fig. 3.46A. Each reading represents the mean value of 6 individual readings

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